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TRANSCRIPTIONAL REGULATION OF LAMB3 BY p53

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

MEGHNA JANI
B.Sc. Biotechnology, University of Mumbai, India, 2005

2008
Wright State University

WRIGHT STATE UNIVERSITY
SCHOOL OF GRADUATE STUDIES

December 12, 2008

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Meghna Jani ENTITLED Transcriptional regulation of LAMB3 by p53 BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Jani, Meghna. M.S., Department of Biochemistry and Molecular Biology, Wright State University, 2008. Transcriptional regulation of LAMB3 by p53.

The p53 tumor-suppressor plays a very important role in the prevention of cancer and it is known that about 50% of all human tumors possess p53 mutations. Although mutations in p53 are most prevalent in human cancers, inactivation of wild-type p53 occurs through many different mechanisms that are independent of p53 mutation or deletion. In an effort to determine novel p53 target genes, our lab employed a microarray method in which p53 was re-activated by RNAi mediated knockdown of Hdm2 and HdmX in MCF7 human breast cancer cell line, harboring wild-type p53 and elevated levels of Hdm2 and HdmX. Gene expression profiling of the RNAi treated MCF7 breast cancer cells led to the identification of Laminin-beta 3 (LAMB3) as a potential transcriptional target of p53. To test this hypothesis, I carried out validation experiments which confirmed the earlier microarray experimental results.

Interestingly, I also found four p53 half binding sites downstream of the LAMB3 promoter. DNA-damage and overexpression studies established that p53 activation unexpectedly did not lead to any transcriptional increase in LAMB3 expression. In contrast, serum deprivation and senescence experiments demonstrated that LAMB3 expression paralleled p21 (a well-known p53 target involved in cell cycle arrest) expression. Taken together, the experimental results suggest that LAMB3 may be a p53 regulated gene, but is not a classical p53 target.

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Dedicated to my parents Upendra Jani and Kamini Jani

I. INTRODUCTION

In the United States of America, cancer is accounted as the second leading cause of death according to the Cancer statistics 2008 of the American Cancer Society - U.S. Mortality 2005 (American Cancer Society. *Cancer Facts & Figures 2008*). The World Health Organization denotes cancer to be the leading cause of death worldwide (World Health Organization). Cancer has been defined as a group of diseases comprising a renegade system of growth in which there is failure to regulate cell division thus leading to uncontrolled growth of cells. The unchecked growth of abnormal cells creates a mass of tissue known as a tumor or neoplasm. This tumor can turn invasive and malignant, encroach other tissues, thus spreading to other parts of the body initiating a chain of uncontrolled cell growth. If left undetected or untreated, the cancer can result into severe illness or death. Although there exists many kinds of cancer, but each shares the common characteristic of uncontrolled cell growth followed by a limitless expansion (American Cancer Society. *Cancer Facts & Figures 2008*; Cancer.gov: National Cancer Institute Web site).

In 1979, two different research groups identified p53 as a cellular protein which was bound to the SV40 T-antigen oncoprotein (Lane & Crawford, 1979; Linzer &

Levine, 1979). Initially, p53 was presumed to be an oncogene, but later was confirmed to be tumor suppressor (Baker, Markowitz, Fearon, Willson, & Vogelstein, 1990; Vogelstein, Lane, & Levine, 2000). p53 functions as the “Guardian of the genome” by tightly controlling cell proliferation or inducing apoptosis (Efeyan & Serrano, 2007; Kuribayashi & El-Deiry, 2008; Lane, 1992; Meulmeester & Jochemsen, 2008; Riley, Sontag, Chen, & Levine, 2008). For this reason it is often inactivated or mutated at the onset of cancer (Vogelstein et al., 2000). This leads to an inactive p53 protein which cannot function as a DNA sequence-specific transcriptional regulator. Hence, this does not allow p53 to transactivate many of its target genes that could have been up-regulated or down-regulated (Oren, 2003) to mediate the various biological effects of p53 such as cell cycle arrest, DNA repair, apoptosis and senescence (Jacobs, Kaplan, & Miller, 2006; Prives & Hall, 1999; Vogelstein et al., 2000; Vousden & Lu, 2002).

Considering its key biological role, p53 is kept under strict regulatory control in an unstressed cell. This control mainly occurs by maintenance of protein stability. In a normal cell, rapid degradation of the p53 protein takes place so that its level is kept low (Harris & Levine, 2005; Jacobs et al., 2006). Hdm2 and HdmX represent two essential negative regulators of p53 (Parant et al., 2001). Hdm2 (the human homolog of Mdm2) is a known ubiquitin ligase and functions by binding to p53 protein, causing ubiquitination of p53 protein and ultimately leading to proteosomal degradation (Harris & Levine, 2005; Honda, Tanaka, & Yasuda, 1997). It also possesses the ability to inhibit p53 transcriptional activity (Klein & Vassilev, 2004). On the other hand, HdmX – the structural homolog of Hdm2, does not possess an ubiquitin ligase activity like Hdm2, but,

it binds to p53 and inhibits the transcriptional activity of p53 (Parant et al., 2001; Shvarts et al., 1996). Under conditions of cellular stress, Hdm2 is also known to promote ubiquitination and degradation of HdmX leading to p53 activation (Pan & Chen, 2003). Hdm2 amplification has been reported in ~10.5 % of 7,711 tumors studied while HdmX was found to be amplified in 9.9 % of 533 tumors and over expressed in 17.2% of 443 tumors (Toledo & Wahl, 2006). Thus, re-activation of p53 achieved by modulating Hdm2 and HdmX levels can be implemented in a potential search for p53 based anticancer therapy strategy.

Within a cell, the p53 protein is present at the hub of an extremely complex network. p53 drives the transcription of many known target genes by recognizing and binding specific DNA sequences along with recruiting both general and specialized transcriptional co-regulators (Laptenko & Prives, 2006). The list of known p53 target genes is large and extensive studies have been performed on them. In spite of this, many putative p53-regulated genes exist which are yet to be studied in detail (Riley et al., 2008). Thus, with a perspective to improve diagnosis and treatment of cancer it is necessary to understand the p53 signaling pathway in-depth. Identification of novel p53 target genes and analysis of their functions will open new arenas leading to unique approaches to inhibit tumor progression. To date, many different methods such as Chip on Chip arrays, Microarray technology and Computational analysis have been used to identify potential p53 regulated genes (Liu, Lagowski, Vanderbeek, & Kulesz-Martin, 2004; Sbisa et al., 2007; Wei et al., 2006). In our lab, microarray technology combined

with RNAi was employed by Dr. Kate Heminger in a pursuit to determine how the loss of Hdm2 and HdmX – two negative regulators of p53 impacted p53 function.

In an effort to determine novel target genes of p53, our laboratory utilized RNAi silencing of Hdm2 and HdmX leading to lower protein levels of both the negative regulators of p53 (Harris & Levine, 2005; Honda et al., 1997; Parant et al., 2001; Riley et al., 2008). This study was undertaken using MCF7 cells, a breast cancer cell line having wild-type p53 and over-expressing Hdm2 and HdmX (Zaika, Irwin, Sansome, & Moll, 2001; Zhou, Frum, Deb, & Deb, 2005). Utilizing Affymetrix microarray technology we systematically evaluated gene expression alterations by activating p53 in a non-genotoxic manner following the loss of Hdm2 or HdmX.

From the analysis of the results of the Affymetrix microarray experiment, Laminin-beta 3 (LAMB3) was identified as a potential p53 transcriptional target gene. LAMB3 is one of the polypeptide chains forming the heterotrimeric glycoprotein Laminin-5, the other two being Laminin-alpha 3 (LAMA3) and Laminin-gamma 2 (LAMC2). Each of the three polypeptides is encoded by three different genes (Akutsu, Amano, & Nishiyama, 2005; Hartwig et al., 2007; Martin et al., 1998; Masunaga et al., 1996; Pulkkinen, McGrath, Christiano, & Uitto, 1995). Laminin-5 is specific to the epithelium and is a major component of anchoring filaments - one of the pivotal hemidesmosomal components at the basement membrane in skin. It plays an important role in the structural relationship connecting the dermis and the epidermis (Akutsu et al., 2005; Calaluce et al., 2004; Rousselle, Lunstrum, Keene, & Burgeson, 1991). The

LAMB3 gene is located on 1q32 chromosome with a size of 29 kb containing 23 exons (Hao, McDaniel, Weyer, Barrera, & Nagle, 2002; Pulkkinen et al., 1995). Mutations in LAMB3 gene are observed in a lethal (Herlitz) variant of Junctional Epidermolysis Bullosa which is a heterogeneous group of diseases characterized by fragility and blistering of skin (Pulkkinen & Uitto, 1999). LAMB3 was also shown to be mutated in generalized atrophic benign epidermolysis bullosa in 2 siblings which was further complicated by multiple squamous cell carcinomas (SCC) (Swensson & Christophers, 1998).

An important discovery was the observation that laminin-5 was down-regulated in breast carcinoma cells (Martin et al., 1998) while there was a loss of protein expression in prostate cancer (Hao et al., 2001). In contrast to these findings, suggesting a link between loss of laminin-5 and breast or prostate cancer the expression of laminin-5 has been detected in invasive epithelial carcinomas at the tumor stromal interfaces of the invading edges (Pyke et al., 1994) and is being recognized as an invasion marker (Katayama & Sekiguchi, 2004). The characteristic increase or decrease in laminin-5 expression in carcinomas obtained from different tissue types may reflect different roles of the molecule in different tissues. Although, there are no studies yet to resolve the observed conflicting results, further analysis will elucidate the role of laminin-5 in the regulatory interplay between migration and stable adhesion.

Additionally, Martinez et al., report that LAMB3 is a target of miR-218 and is down-regulated at the transcriptional level in cervical carcinoma cell lines containing

integrated Human papilloma virus type 16 (HPV-16). They also state that down-regulation of miR-218 by the E6 oncogene of HPV-16 along with overexpression of LAMB3 may promote viral infection of surrounding tissue and contribute to eventual tumorigenesis (Martinez et al., 2008). In a study by another group, the E6 protein has been shown to promote ubiquitination and proteosomal degradation of p53 protein (Thomas, Pim, & Banks, 1999). Taking these findings into account, it will be interesting to understand the relation between p53 and LAMB3, which will help gain insights into the role of p53 in cancer prevention.

In the present study, I analyzed LAMB3 which was detected as a potential p53 regulated gene. From the microarray experimental data it appeared that LAMB3 mRNA levels were induced in MCF7 cells where Hdm2 or HdmX were knocked down. Thus, I performed validation experiments using RNAi targeting Hdm2 and HdmX and confirmed these results namely that LAMB3 was induced upon loss of Hdm2 and HdmX. Consequently, LAMB3 expression was monitored in response to cellular stress by DNA-damage. Except for two cell lines, LAMB3 did not respond to the inflicted stress. Overexpression of p53 also did not lead to an increase in LAMB3 transcript level. The inability to induce LAMB3 following p53 activation by genotoxic stress or through overexpression suggested that LAMB3 was not a classic p53 regulated gene. Since the loss of Hdm2 and HdmX in MCF7 cells resulted in a cell cycle arrest (Heminger et al., manuscript submitted), I evaluated LAMB3 response to cell-cycle arrest by carrying out serum deprivation studies in MCF7 cells. LAMB3 expression paralleled p21 (a known p53 target gene) expression which showed a decrease. It is now known that although the

final effect of anti-proliferative signals is growth arrest, the cellular processes initiated by the cell cycle arrest are dependent on the nature of the extracellular signals and the cell type receiving them (Wainwright, Lasorella, & Iavarone, 2001). This maybe the reason of differential response of both LAMB3 and p21 to cell cycle arrest induced in two different ways i.e., increased upon knockdown of Hdm2 or HdmX and decreased upon serum deprivation. In addition, since Ruth Sager (Sager, 1991) demonstrated senescence as a mode of tumor suppression, I set out to look at the effect on LAMB3 upon the onset of ras induced senescence as a mode of stress. From the experimental data it was seen that LAMB3 transcript levels correlated to p21 mRNA levels which showed an increase upon the onset of ras induced senescence.

II. MATERIALS AND METHODS

Cell Lines and Culture

The human tumor cell lines, MCF7 (breast adenocarcinoma), HCT116 +/+ p53 and HCT116 -/- p53 (colorectal carcinoma), H1299 (non-small cell lung carcinoma), SaOS2 (epithelial-like osteosarcoma), U2OS (osteosarcoma), 293FT (embryonic kidney) were purchased from American Type Culture Collection (ATCC). These cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Cellgro-Mediatech, Inc.) supplemented with 10% Bovine Growth Serum (BGS; Hyclone) or 10% Newborn Calf Serum (NCS; Invitrogen) and 10 µg/mL Gentamicin (Invitrogen). MCF10A (Human immortalized breast epithelial) and 76N tert (Human immortalized mammary epithelial) were kind gifts from Dr. Vilma Band, Northwestern University, IL and were grown in Mammary Epithelium Basal Medium (MEBM; Lonza) supplemented with MEGM[®] SingleQuots[®] (Lonza) containing Bovine Pituitary Extract, hEGF, Insulin, Hydrocortisone, Gentamicin. LnCaP (Human prostate carcinoma) were purchased from ATCC and maintained in RPMI 1640 (Cellgro-Mediatech, Inc.) with 10% Fetal Bovine Serum (FBS; Invitrogen) and 1% Penicillin and Streptomycin (Invitrogen). IMR90 (Human diploid lung fibroblast cell line) was obtained from ATCC and maintained in DMEM supplemented with 10% Fetal Bovine Serum and 10 µg/mL Gentamicin.

Antibodies and Reagents

Monoclonal p53 antibody (Ab-6; Calibiochem), LAMB3 antibody (Anti-Kalinin B1; Transduction Laboratories), beta-actin antibody (Sigma-Aldrich®), and Polyclonal p21 antibody (Santa Cruz Biotechnology, Inc.) were used as mentioned. Horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibodies (Promega) were used along with Super Signal® West Pico Chemiluminescent substrate (Thermo Scientific) to detect proteins by chemi-luminescence. Doxorubicin hydrochloride (Sigma-Aldrich®) and Bleomycin (Sigma-Aldrich®) were used as indicated. siGENOME duplex RNA targeting *p53*, *hdm2*, *hdmX* mRNA and a non-targeting control siRNA were purchased from Dharmacon Research, Inc. and 20 µM stock solution was made according to the manufacturer's protocol. Aliquots for each siRNA were stored at -20°C. The si transfection was carried out using Lipofectamine 2000 (Invitrogen) and/or Oligofectamine (Invitrogen) as described below.

siRNA transfection

Reverse transfection with siRNA was carried out on MCF7 cells at the time of seeding followed by a second round of transfection 24 hours later. Triple transfection with siRNA was carried out for all other cell lines for three consecutive days starting 24 hours after cells were plated. Seeding of cells was done in antibiotic free growth media (DMEM–no antibiotic). The appropriate volumes of each component essential for setting up a 100 nM siRNA transfection in the various cell culture vessels are listed in Table 1.

	6-well plate	6-cm dish
Cell Number	200,000/well	700,000/dish
Volume of siRNA + SFM	5 μ L siRNA + 180 μ L SFM	7.5 μ L siRNA + 177.5 μ L SFM
Volume of Oligofectamine + SFM	3 μ L lipid + 112 μ L SFM	4.5 μ L lipid + 110.5 μ L SFM
Total Volume	1.0 mL	1.5 mL

Table 1: Required volumes of siRNA, lipid and serum free media to perform siRNA transfection. The above table indicates the appropriate volumes of each component required to setup a 100 nM siRNA transfection in the respective cell culture vessels.

Reverse transfection

In the case of reverse transfection, 100 nM siRNA with Lipofectamine 2000 (Invitrogen) in antibiotic free DMEM containing 1% BGS/NCS was used at the time of seeding (see Table 1 for details). The Lipid-media mixture and the siRNA-media mixture were incubated at room temperature for 30 minutes before transfection. To the required number of cells in media, 300 μ L of siRNA-Lipofectamine mixture was added and DMEM containing 1% BGS/NCS was used to adjust the volume to 1 mL. Similarly the volumes of siRNA, cells, Lipofectamine 2000 and DMEM containing 1% BGS/NCS was adjusted such that the final concentration of siRNA was 100 nM and the final volume was 1.5 mL per 6 cm dish. Media was aspirated after the cells were incubated for 5 hours at 37°C in a 5% CO₂ incubator (humidified atmosphere of 95% air, 5% CO₂) and then re-fed with DMEM containing 10% BGS/NCS. Twenty four hours after the first transfection, cells were transfected with same amount of siRNA along with Oligofectamine (Invitrogen) in serum free media. Following a four hour incubation at 37°C in a 5% CO₂ incubator, an equal volume of DMEM containing 20% BGS/NCS was added to each well/dish. Total RNA extraction was performed using the RNA extraction kit (RNeasy[®] mini kit; Qiagen) (Appendix I), twenty four hours after the final siRNA transfection.

Triple transfection

Triple transfection was performed by plating cells twenty four hours prior to the first siRNA transfection. Plating density, dilutions of oligofectamine and amount of

siRNA are as given below in Table 1. The mixture of siRNA and oligofectamine was prepared in serum free media and the remaining method was similar to the protocol followed for day 2 of the reverse transfection. The previously plated cells were washed with serum free media and 700 μ L serum free media was added to each well of a 6-well plate. Then 300 μ L of siRNA-oligofectamine mixture was added to each well, thus leading to a total volume of 1 mL and a siRNA concentration of 100 nM per well. For a 6 cm dish the same volumes of reagents were used as for the reverse transfection with the total volume adjusted to 1.5 mL per dish. The plates were incubated for four hours at 37°C in a humidified atmosphere of 95% air and 5% CO₂ after which 1 mL of DMEM containing 20% BGS/NCS was added to each well without aspirating the transfection mix. The same transfection was repeated at 24 hours and 48 hours following the first transfection. Total RNA extraction was performed using the RNeasy[®] mini kit (Qiagen) (Appendix I), twenty four hours after the last siRNA transfection.

Protein Assay, SDS-PAGE and Western Blot Analysis

Cells were scraped from each dish/well along with media and collected in a sterile 1.7 mL eppendorf tube or sterile 15 mL tube. For transfections, cells were scraped 24 hours following the last transfection unless otherwise noted. Centrifugation was then performed at room temperature and 1000 rpm for 4-5 minutes to pellet the cells. The media was aspirated and the pellet was re-suspended in a volume of 1 mL DPBS and transferred into a 1.7 mL eppendorf tube. This mixture was centrifuged in a refrigerated centrifuge (4°C) at 14,000 rpm for 2-3 minutes. The supernatant was aspirated, pellet

frozen at -80°C for 30 mins and a whole cell extract was prepared by re-suspending the cell pellet in 50 μL cold Single Lysis Buffer (50 mM Tris pH 8.0, 150 mM sodium chloride, 0.25% Nonidet P-40) containing 1% protease inhibitor cocktail (PIC; Sigma-Aldrich[®]). Cells were lysed by pipetting up and down. To allow complete protein extraction the cell pellet-Single Lysis Buffer-PIC mixture was placed on ice for 30 minutes. The insoluble material was pelleted in a refrigerated centrifuge (4°C) at 10,000 rpm for 5 minutes. The supernatant containing the soluble protein fraction was transferred to an eppendorf tube and stored at -80°C .

The total cell protein was analysed using the Bradford assay. One micro-liter of the soluble protein fraction was mixed with 799 μL sterile distilled water (SDW) followed by addition of 200 μL Bradford reagent (Bio-Rad Inc.). Standards were set up using Bovine Serum Albumin (BSA) protein, ranging from 0 $\mu\text{g}/\mu\text{L}$ to 14 $\mu\text{g}/\mu\text{L}$ of BSA with SDW followed by addition of 200 μL Bradford reagent. The optical density (OD) was measured at 595 nm using a spectrophotometer. A standard curve was generated by plotting the OD of the BSA standards against the BSA concentration. The cell extract protein concentrations were calculated by extrapolating the absorbance values of the samples against the standard curve of BSA.

For SDS-PAGE, 100 μg -200 μg of protein was mixed with Single Lysis Buffer-loading buffer (used to balance the volume of sample loaded onto the gel) and an equal volume of 2X SDS-sample loading dye (60 mM Tris, pH 7.6, 2% SDS, 10% glycerol, 5%

β -mercaptoethanol, and 1% bromophenol blue), heated at 65°C-70°C for 2-3 minutes and loaded onto a Sodium Dodecyl Sulphate (SDS) 10% polyacrylamide gel. Protein markers (Invitrogen) were loaded along side the protein samples. SDS running buffer (1X = 25 mM Tris pH 8.3, 250 mM glycine, 0.1% SDS) was used and the gel run at 150 constant volts for 4-5 hours. Using a Transblot system (Bio-Rad, Inc.) proteins were transferred from the gel onto a polyvinylidene difluoride (PVDF) membrane (Millipore) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3), overnight at 50 volts.

Following the transfer, the membrane was blocked by incubating it in 40-70 mL blocking buffer (1X Phosphate Buffered Saline (PBS), 5% non-fat dry milk, 0.2% Tween-20) for 1 hour at room temperature or overnight at 4°C on a rotatory platform shaker. Next, using specific primary antibodies at 1:200-1:5000 dilution in 1X PBS containing 0.5% non-fat dry milk and 0.025% Tween-20 (Dilute Blocking Solution), immunoblotting was carried out at room temperature for 1 hour on a rocker or overnight on a rocker at 4°C. Next, the membrane was washed three times for 15 minutes each with Dilute blocking solution on a platform shaker. The membrane was then incubated for 1 hour at room temperature on a rocker with either a goat anti-mouse or goat anti-rabbit HRP-conjugated antibody (Promega) with a dilution of 1:5000 in dilute blocking solution. Afterwards, the membrane was washed three times in dilute blocking solution for 15 minutes each. Immunoreactivity was identified by soaking the membrane in 10 mL of super signal chemiluminescent reagent for 3-4 minutes and HRP induced chemiluminescence was visualized on FUJI FILM LAS 3000 CCD camera. For re-probing the blot, the membrane was first incubated in western stripping buffer (25 mM

glycine, 1% SDS, pH 2.0); two times for 20 minutes each on platform shaker at room temperature. This “stripped” membrane was washed extensively with PBS and then immunoblotted using another antibody following the method stated above.

Serum Deprivation/Flow Cytometry

For serum deprivation experiments, cells were plated at a density of 2×10^5 cells for the asynchronous 24 hour condition, 4×10^5 cells for asynchronous 48 hour and serum free 24/48 hour conditions in a 6 cm dish. Trypsinization of cells was done 24 or 48 hours after depriving cells of serum. For trypsinization, the media from the plates was transferred to a 15 mL sterile tube. Afterwards, the cells were trypsinized with 1 mL of 0.25% Trypsin/EDTA per 6 cm dish. The trypsinized cells were collected in the respective 15 mL sterile tube. The dish was rinsed with 2 mL DPBS and this was transferred into the respective 15 mL sterile tube. The tube was then completely filled with DPBS. Centrifugation was performed at 1200 rpm for 5 minutes to pellet the cells. DPBS was then decanted by inverting the tube and the cells were re-suspended in the small amount of remaining DPBS by flicking the tube. Each tube was continuously vortexed while adding 2 mL of 70% ethanol in a drop-wise manner. The tubes were stored at -20°C until flow analysis was performed. At the time of flow analysis; the tubes were spun at 1200 rpm for 5 minutes to pellet cells. Ethanol was decanted and the cells were re-suspended in the remaining amount of liquid. A mixture was prepared by adding 1 mL RNase A (1 mg/mL) and 9 mL of DPBS. To each tube 500 μL of this mixture was added and the tubes were allowed to incubate at 37°C for 20 minutes. To stain the cells,

50 μ L of Propidium iodide (50 μ g/mL; Sigma Inc.) was added to each tube. The tubes were covered with foil to protect them from light and placed on ice for 30 minutes. Next, the cell suspensions were transferred to flow tubes. Flow cytometry was performed (channel FL2) on a FACSCAN (Becton Dickinson). Using the Modfit LT 3.0 software (Verity Software House, Inc.), the percentage distribution of cells in G1, S and G2/M phase was acquired with the help of Dr. Steven Berberich.

Quantitative RT-PCR

For RNA isolation, cells were washed once with DPBS. Total RNA extraction and purification was done using the RNeasy[®] mini kit (Qiagen) following the manufacturer's protocol (Appendix I). The quality and quantity of RNA was estimated using the Agilent Bioanalyzer (Agilent Technologies) and RNA Nano Chips (Agilent).

cDNA was made for each RNA sample by reverse transcribing 0.5 μ g total RNA with random hexamers using the Taqman Reverse Transcription Kit (Applied Biosystems), as per manufacturer's protocol (Appendix II). The Reverse Transcription (RT) reaction was performed in the Applied Biosystems Gene Amp PCR System 2700 (thermal cycler) in a reaction volume of 25 μ L. The temperature and time setup for the RT reaction employed in the thermal cycler are as follows: - initial 10 minute incubation at 25°C, 30 minute RT step at 48°C, 5 minute inactivation step at 95°C, and an infinite hold at 4°C. On the completion of the RT reaction, the cDNA was diluted 1:9 by adding 200 μ L sterile DNase-free water to each sample and stored at -20 °C.

The Quantitative real-time PCR (RT-qPCR) reaction was carried out in a 96-well microtiter plate format using the ABI Prism 7900HT sequence detection system. For each well of the 96-well microtiter plate, 1 μ L (2.5 ng) of cDNA, 8 μ L of DNase-free water, 10 μ L of 2X TaqMan Universal PCR Master mix, and 1 μ L of Assay-on-Demand Gene Expression product (Applied Biosystems, containing forward and reverse primers and a fluorescent Taqman probe) designed and optimized for gene of interest, were added to make the total volume to 20 μ L. The conditions applied for the RT-qPCR are as follows: - 2 minute hold at 50°C, a 10 minute hold at 95°C, and 40 cycles of a 15 second 95°C denaturing step and a 1 minute 60°C anneal and elongation step. All the cDNA samples were analyzed in triplicates for each required target gene as well as GAPDH (endogenous control). The Relative Quantification (RQ) i.e. the fold change relative to the control sample was calculated based on a PCR efficiency of two and normalized to GAPDH RNA levels by using the SDS 2.0 software (Applied Biosystems). The CT values which differed by one full CT from the mean value of the other two replicates, known as outlier cycle threshold (CT) were removed. RQ values were used for plotting the graphs, while the error bars represent variation as the 95% confidence interval about the mean RQ value for each data point.

Senescence Assay

For the senescence assay, IMR90 cells were seeded at 40,000 cells per well of a 6 well plate, twenty-four hours prior to infection with the lentivirus. The transduction of IMR90 cells with lentivirus expressing Ras, EGFP or the combination were employed for

the experimental setup. Each treatment condition was performed in duplicate such that both RNA extraction and quantitation of senescence can be performed at the same time. On the day of infection, the supernatant containing lentivirus (1 mL) was thawed and diluted in a small volume of Complete Media (DMEM with 10% FBS and 10 μ g/mL Gentamicin (antibiotic)) with 4 μ g/mL polybrene. The media was aspirated from the cells, and the diluted virus was added to each well to achieve a MOI (Multiplicity of Infection = Number of infectious viral particles per cell) of five. Cells were incubated at 37°C unless and otherwise stated. On the next day, infection cocktails were removed from each well and replaced with 2 mL complete media. After twenty-four hours, 6 μ g/mL blasticidin diluted in complete media was added to each well for the selection process. The cells were re-fed with 6 μ g/mL blasticidin diluted in complete media every 2-3 days for 7 days. The control well with no viral infection stayed in complete media with no selection for the duration of the experiment. To assess senescence, the cells in one well per condition were assayed for beta-galactosidase activity. This was done using the Senescence β -Galactose Staining Kit (Cell Signaling Technology) following the manufacturer's instructions (Appendix III). For each condition, images were captured by an Olympus IX70 microscope and total number of cells, number of blue cells (senescent cells) and number of green cells (EGFP infected cells) was scored. Simultaneously, from the second well for each condition (non-stained, but selected) the antibiotic resistant cells (infected cells) were harvested for RNA extraction and used for further testing. The percent senescence was calculated by dividing the percentage of blue cells to the percentage of total number of cells. Transduction efficiency was determined by dividing the percent of EGFP infected cells by the percentage of total number of cells.

DNA Damage Experiments

Cells within the range of 100K-250K per plate were plated twenty-four hours prior to treatment with the DNA damaging agents Doxorubicin or Bleomycin. The next day, cells were treated with increasing doses of the DNA damaging agents as specified in the results. Twenty-four hours post treatment; total RNA was harvested and used for further testing.

For the time course experiment, MCF7 and HCT 116 cells either containing wild-type p53 or lacking p53 were plated in 6 cm plates at a density of 200K cells/plate. Twenty-four hours after plating, the cells were treated with 0.25 µg/mL doxorubicin and RNA was extracted at 0 hr, 6 hr, 12 hr and 24 hr. RT-qPCR was performed using the Taqman based system (Applied Biosystems) on total RNA from each time point.

Plasmid Transfections

The tumor cells U2OS (250K cells), SaOS2 (70K cells), H1299 (80,K cells) and HCT 116 -/- p53 (300K cells) were seeded in 6 cm plates with DMEM supplemented with 10% Bovine Growth Serum and 10 µg/mL Gentamicin [Complete Media], 24 hours prior to transfection. pcDNA3.1-p53, pcDNA3.1-p63γ-GST and pcDNA3.1-p73α expression constructs were used for the exogenous expression of p53 family members. pcDNA3.1-p63γ-GST and pcDNA3.1-p73α expression constructs were kind gifts from Dr. Madhavi Kadakia. The required amount of each plasmid DNA was aliquotted into

eppendorf tubes and a mammalian expression vector encoding Green Fluorescent Protein (GFP) was added to each tube. pcDNA3.1 was used to bring the total DNA for each transfection to 1 µg. The Lipid:DNA ratio used for the transient transfection was 3:1. For SaOS2, H1299, U2OS and HCT116 +/- p53 cells, 400 µL of serum free media (SFM) was added to the aliquotted DNA (0.125 µg p53, 0.25 µg p53, 1 µg each of p63γ and p73α) and tubes were vortexed. Afterwards, 12 µL of Lipofectamine 2000 (Invitrogen) was added to 400 µL of SFM in an eppendorf tube and mixed by inversion. The DNA + SFM was combined with the Lipofectamine + SFM, mixed by gentle inversion and allowed to incubate at room temperature for 25 minutes. The media on the 6 cm plates was aspirated and 800 µL of SFM was added onto the plate along with 200 µL of the DNA–Lipofectamine 2000 mixture. The plates were then incubated at 37°C in a 5% CO₂ incubator for five hours. After the incubation, media was aspirated and 2 mL of fresh complete media was added. Total RNA was extracted 24 hours post transfection and used for RT-qPCR.

Primer Selection and SYBR[®] Green PCR Assay

Primers were generated for the two mRNA isoforms; NM_001017402 and NM_000228 of LAMB3 listed on the UCSC genome browser (<http://genome.ucsc.edu/>) assembly March 2006. The primers specific for each of the two isoforms, common to both were obtained from Integrated DNA Technologies and are as listed: - NM_001017402 (forward, 5'-AAGGTCCTTTCTGGGGATCA-3'; reverse, 5'-CTTGTTGGGCATGCAGGAG-3'), NM_000228 (forward, 5'-

CCCACATTCAAGAGGAGCTT-3'; reverse, 5'-TGGTCTCATCTTCAGCCAAT-3') and both (forward, 5'-GTGGACTGACCAAGCCTGA-3'; reverse, 5'-CGGTGACTGTAGTAGTTGTGAGG -3') while for GAPDH (normalization control) (forward, 5'-ATGTTTCGTCATGGGTGTGAA -3'; reverse, 5'-GGTGCTAAGCAGTTGGTGGT-3') were obtained from Invitrogen. Each primer was diluted with water to get a concentration of 1.5 pmol/μL. For the SYBR[®] Green PCR Assay, the total RNA was first treated with DNase (DNA-free kit; Ambion Inc.) to obtain DNA free RNA following the manufacturer's instructions (Appendix IV). Each RNA sample was then converted to cDNA by reverse transcribing each RNA sample with random hexamers using the Taqman Reverse Transcription Kit (Applied Biosystems), as per manufacturer's protocol (Appendix II). The SYBR[®] Green PCR reaction was carried out in a 96-well microtiter plate format using the ABI Prism 7900HT sequence detection system using the SYBR[®] Green PCR core reagent (Applied Biosystems) kit. Table 2 A and B lists the Mastermix A and B respectively, to be prepared for the one step PCR reaction.

The mastermix (A) was prepared for each of the condition in the experiment, while the mastermix (B) was prepared for each of the primer involved in the study. From the mastermix (A) - 9.5 μL and from mastermix (B) - 15.5 μL was added to the 96 well plate as per the condition. The thermal cycling conditions used in the ABI Prism 7900HT sequence detection system for the SYBR[®] Green Assay are as follows: - 2 minute hold at 50°C, a 10 minute hold at 95°C, and 40 cycles of a 15 second 95°C denaturing step, a 30 second hold at 51°C followed by a 1 minute 68°C anneal and elongation.

(A)

	Volume (μL) for one 25 μL reaction
10X SYBR [®] Green buffer	2.5
25 mM MgCl ₂	3.0
12.5 mM dNTP mix	2.0
Template (cDNA i.e. 0.04 μg RNA)	2.0

(B)

	Volume (μL) for one 25 μL reaction
Forward Primer	1.0
Reverse Primer	1.0
Ampli Taq Gold (5U/ μL)	0.125
AmpErase UNG (1U/ μL)	0.25
dH ₂ O	13.125

Table 2: Required volumes of components to perform a 25 μL reaction for SYBR[®] Green PCR Assay. (A) Volumes of 10X SYBR[®] Green buffer, MgCl₂, dNTP mix and cDNA template composing Mastermix A and (B) Volumes of Forward and Reverse primers, Ampli Taq Gold, Amp Erase UNG and Distilled water composing Mastermix B. (A) and (B) indicate the appropriate volumes of components required to setup a 25 μL reaction for SYBR[®] Green PCR Assay.

III. RESULTS

Up-regulation of known p53 target genes upon RNAi mediated knockdown of Hdm2 and HdmX using Affymetric GeneChips.

DNA Microarrays (Affymetrix GeneChips) were used to investigate the gene expression profiles in MCF7 cells which were transfected with siRNAs targeting Hdm2, HdmX and p53. In the method applied for the study, p53 was activated in a non-genotoxic manner by eliminating either of its two negative regulators Hdm2 and HdmX. This resulted in a list of known p53 target genes that were up-regulated upon p53 activation due to knockdown of Hdm2 or HdmX. A list of thirteen such genes is shown in Figure 1. Knockdown of Hdm2 or HdmX resulted in increased transcript levels of these genes while decreased transcript levels were seen in the knockdown of p53 or the combination of HdmX and p53 as compared to siControl (non-targeting siRNA) for many of these genes. During the course of analyzing the results from the microarray experiment, several genes were identified as putative p53 target genes because they showed a similar pattern of expression to the known p53 target genes. Laminin-beta 3 (LAMB3) appeared to be a putative p53 target gene and its expression values are given in Table 3. Thus, the increase seen in LAMB3 expression in MCF7 cells upon siHdm2 and siHdmX along with a decrease upon sip53 and the combination – siHdmX + sip53 formed a strong basis to propose the hypothesis that LAMB3 is a novel and classical p53 transcriptional target gene.

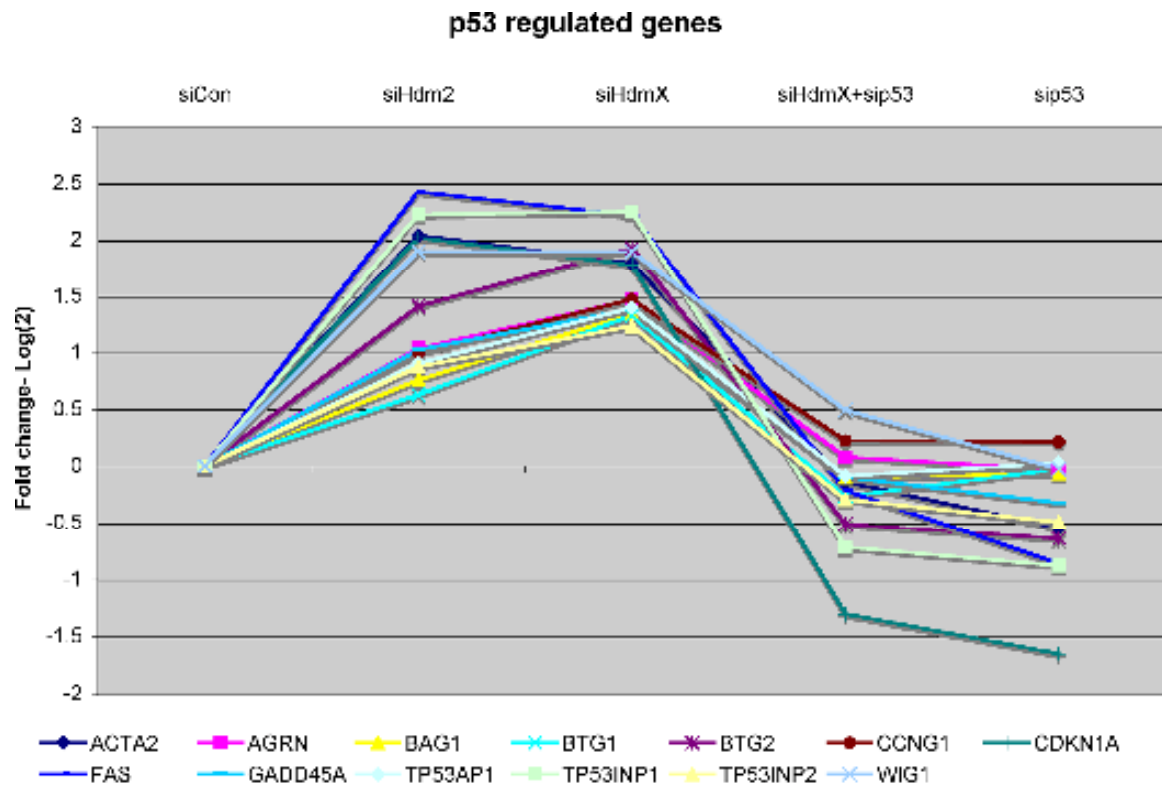
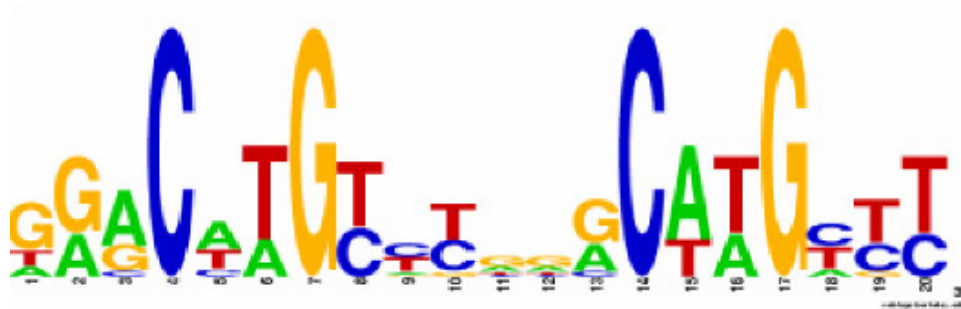


Figure 1: Expression of 13 known p53-regulated genes induced upon knockdown of two p53 negative regulators – Hdm2 or HdmX from the Affymetrix GeneChip analysis. X-axis (top) represents names of siRNA targeting specified genes and Y-axis represents average fold change (log 2) relative to siControl for each of the genes. This experiment was performed by Dr. Kate Heminger.

Treatment type	Relative Expression
	LAMB3
Mock	0.669
siCon	0.748
siHdm2_(A)	1.618
siHdm2_(B)	1.147
siHdmX_(A)	1.165
siHdmX_(B)	1.221
siHdmX(A)+sip53	0.302
sip53	0.285

Table 3: Relative Expression of LAMB3 in the Affymetrix GeneChip using the specified siRNA combination. Two different siRNA target sequences (A) and (B) for Hdm2 and HdmX were used to target the same gene. The target sequence (A) was used for further analysis. This experiment was performed by Dr. Kate Heminger.

(A)



(B)

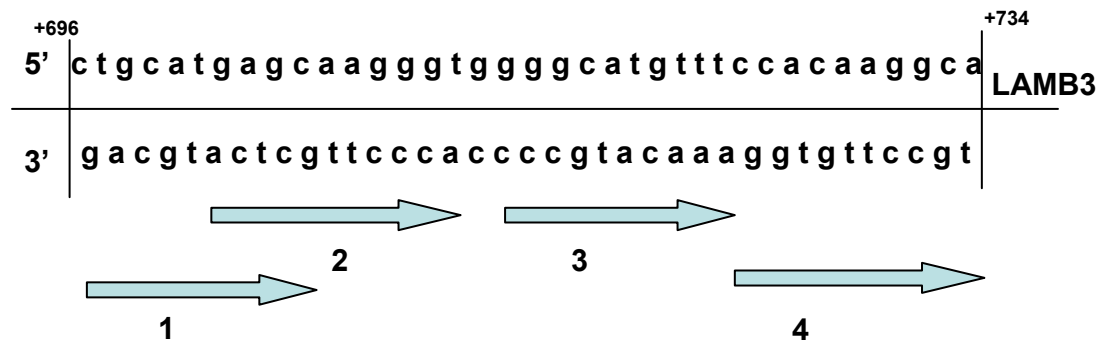


Figure 2: Four half binding sites for p53 located in the first intron of LAMB3. (A) WebLogo multiple sequence alignment of p53 binding sites. Individual base letter height indicates level of conservation within each binding site position. Consensus binding site for p53 - www.biomedcentral.com/1471-2164/8/139/figure/F9. **(B)** Four half binding sites for p53 are seen in the first intron of LAMB3 using TOUCAN and Lasergene's Sequence Builder starting at +696 bp downstream of the transcription start site and spans a 37 bp region. The percentage match of the half binding sites to the consensus binding site for p53 is 1) ctgcatgagc - 60%, 2) gagcaagggt - 80%, 3) gggcatgttt - 100%, 4) ccacaaggca - 60%.

Four p53 half binding sites located in the Intron of LAMB3.

Since the Affymetrix results of LAMB3 gene expression were consistent with LAMB3 being a p53 regulated gene, I used the TOUCAN program (Aerts et al., 2005) to determine if a p53 response element could be identified in or near the LAMB3 promoter. TOUCAN showed two putative p53 half binding sites – one in the promoter and one in the first intron of LAMB3 (data not shown). The p53 half binding site within the first intron of LAMB3 showed a 100% consensus to the known half binding site for p53 (el-Deiry, Kern, Pietenpol, Kinzler, & Vogelstein, 1992). The consensus binding site for p53 is shown in a WebLogo multiple sequence alignment of p53 binding sites (Figure 2A) (Brynczka, Labhart, & Merrick, 2007). Following this, I used the Sequence Builder program of Lasergene and manually screened the region for more p53 half binding sites. Figure 2B gives the detailed region of the first intron with arrows indicating the p53 half binding sites. The binding sites and their percent match to the p53 consensus are as follows: - 1) ctgcatgagc – 60%, 2) gagcaagggt – 80%, 3) gggcatgttt – 100%, 4) ccacaaggca – 60% (Figure 2B).

Induction of LAMB3 transcript levels upon p53 activation by RNAi mediated knockdown of Hdm2 and HdmX in MCF7, MCF10A and 76N tert cell lines.

From the analysis of the Affymetrix GeneChip results it appeared that LAMB3 could be a possible transcriptional target of the p53. To confirm the Affymetrix microarray results and to test the hypothesis that LAMB3 is a transcriptional p53 target gene, the RNAi knockdown experiment in MCF7 cells was repeated and LAMB3 RNA levels quantified by RT-qPCR using Taqman probes. Reverse transfection with siRNA

was performed on MCF7 cells (See Materials and Methods for details) using the following combinations of si constructs – siControl, siHdm2, siHdmX, siHdm2 + siHdmX, sip53 and siHdmX + sip53. Twenty-four hours after the last transfection total RNA was isolated and quantified using an Agilent bioanalyzer. Reverse transcription was carried out using the RNA samples followed by Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR). The analysis of RT-qPCR showed that LAMB3 transcript levels were just slightly increased upon knockdown of Hdm2 (~25%) and HdmX (~50%) yet induced about 2 fold following the simultaneous knockdown of Hdm2 and HdmX (siHdm2 + siHdmX) (Figure 3A). Knockdown of p53 in MCF7 cells showed a statistically significant decrease (~25%) in LAMB3 transcript levels as compared to siControl. The combined knockdown of p53 and HdmX did not show any change in the transcript levels of LAMB3 (Figure 3A). To confirm that the RNAi knockdown was effective the mRNA levels of p21, a known p53 regulated gene were also assessed using RT-qPCR. When compared to siControl, transcript levels of p21 were induced ~4 fold upon knockdown of Hdm2 and ~3.5 fold upon knockdown of HdmX (Figure 3B). The combined knockdown of Hdm2 and HdmX showed a ~12 fold induction of p21 transcript levels while the knockdown of p53 led to a statistically significant reduction of the p21 transcripts. In addition, the knockdown of p53 and HdmX did not significantly affect p21 mRNA levels (Figure 3B).

In an effort to examine how the knockdown of Hdm2, HdmX or p53 affected p53 transcriptional activity in MCF7 cells, luciferase assay was employed. Loss of either of the Hdms led to an increase in p53 transactivation which was completely eliminated

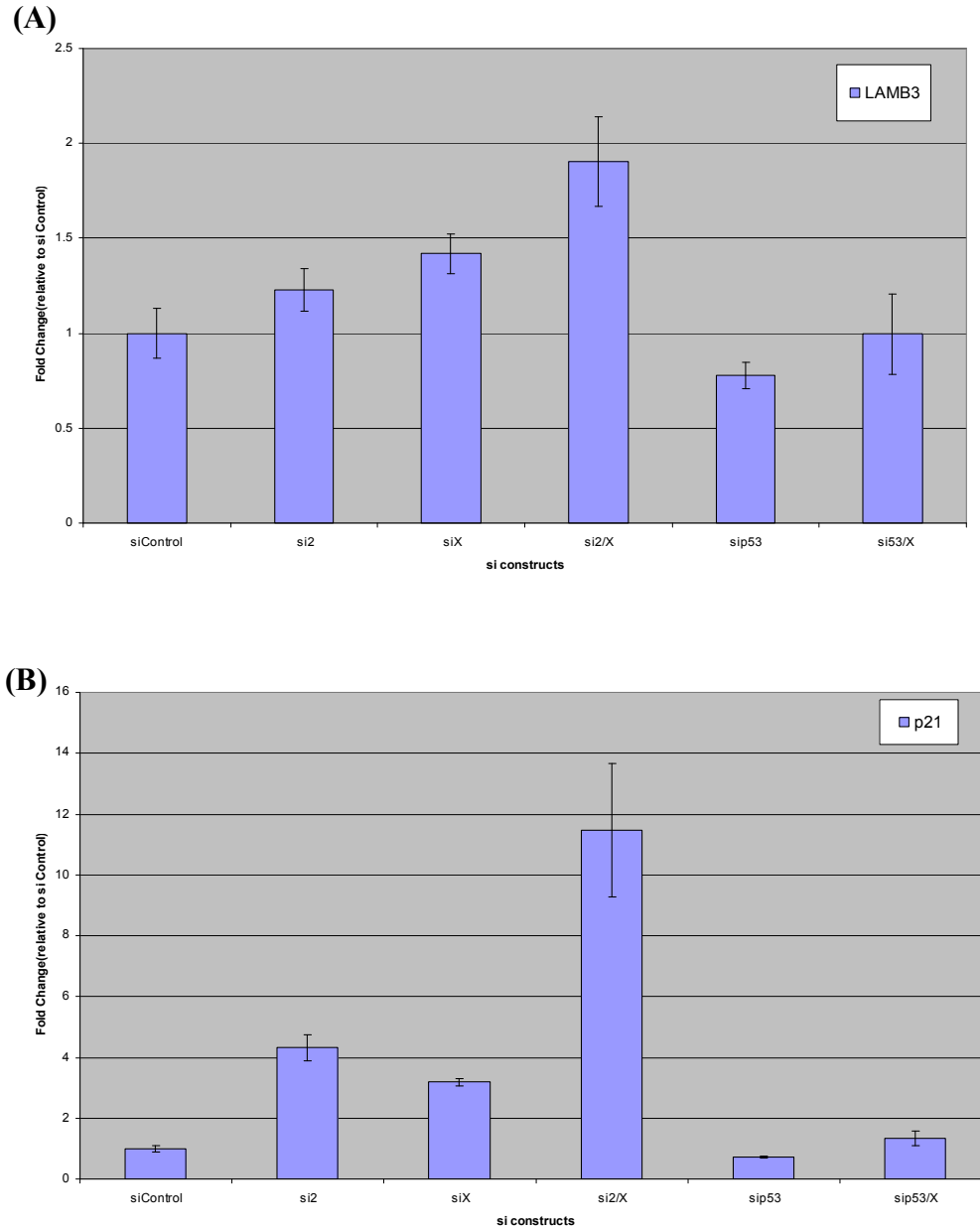


Figure 3: LAMB3 and p21 are induced by knockdown of Hdm2/HdmX using siRNA treatment in MCF7 cells. Reverse transfection was carried out on MCF7 cells for the knockdown of Hdm2, HdmX, Hdm2 and HdmX, p53 or HdmX and p53 as described in Materials and Methods. Twenty four hours later RNA was extracted and reverse transcription was performed. Following this, Real-time PCR was carried out in triplicate to quantify the expression of (A) LAMB3 and (B) p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to siControl and normalized to GAPDH expression. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

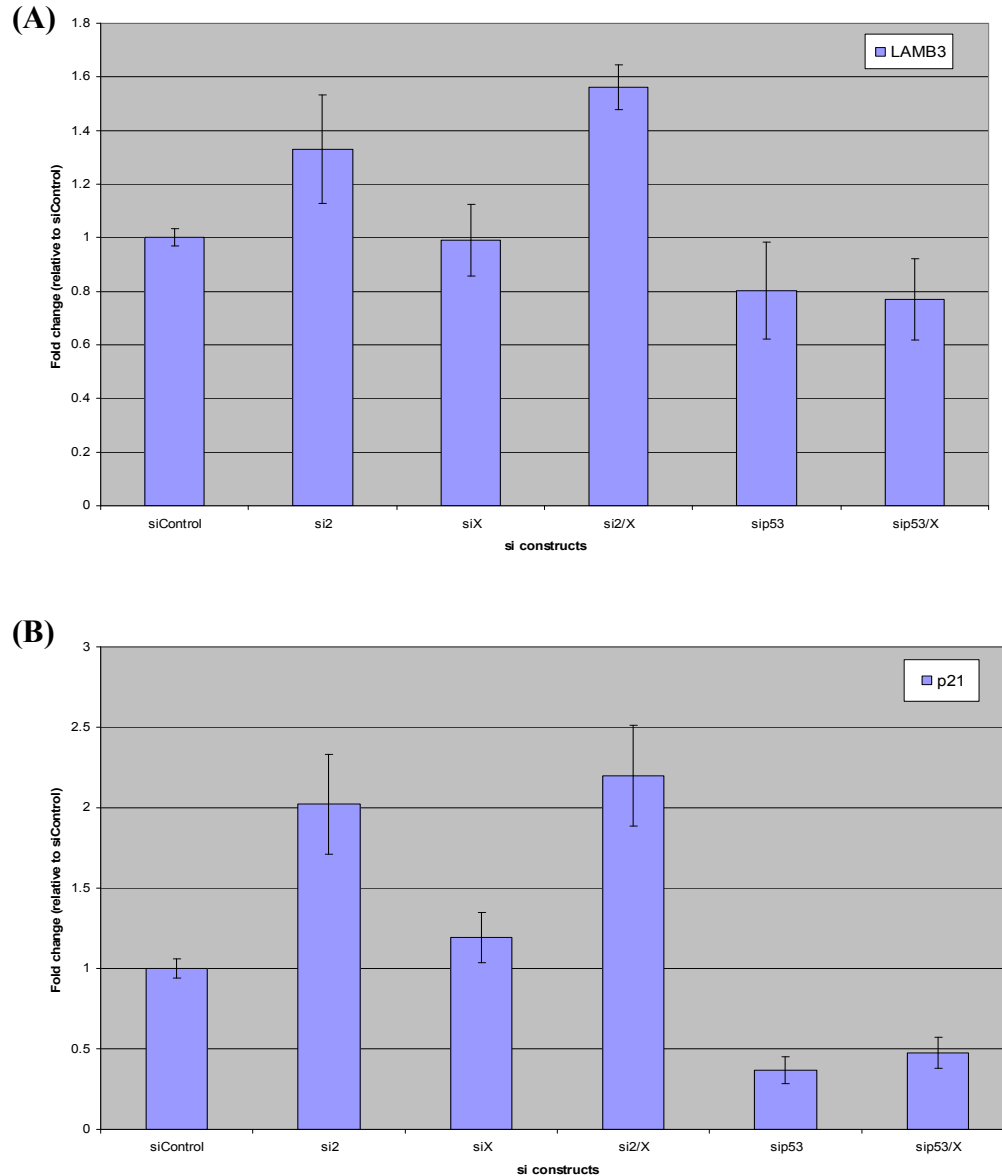


Figure 4: Induction of LAMB3 and p21 by knockdown of Hdm2/HdmX using siRNA treatment in MCF10A cells. Triple transfection was carried out on MCF10A (Human immortalized breast epithelial) cells for the knockdown of Hdm2, HdmX, Hdm2 and HdmX, p53 or HdmX and p53 as described in Materials and Methods. Twenty-four hours after the last siRNA transfection RNA was extracted and reverse transcription was performed. Following this, Real-time PCR was carried out in triplicate to quantify the expression of **(A)** LAMB3 and **(B)** p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to siControl, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars. This experiment was performed by Dr. Steven Berberich.

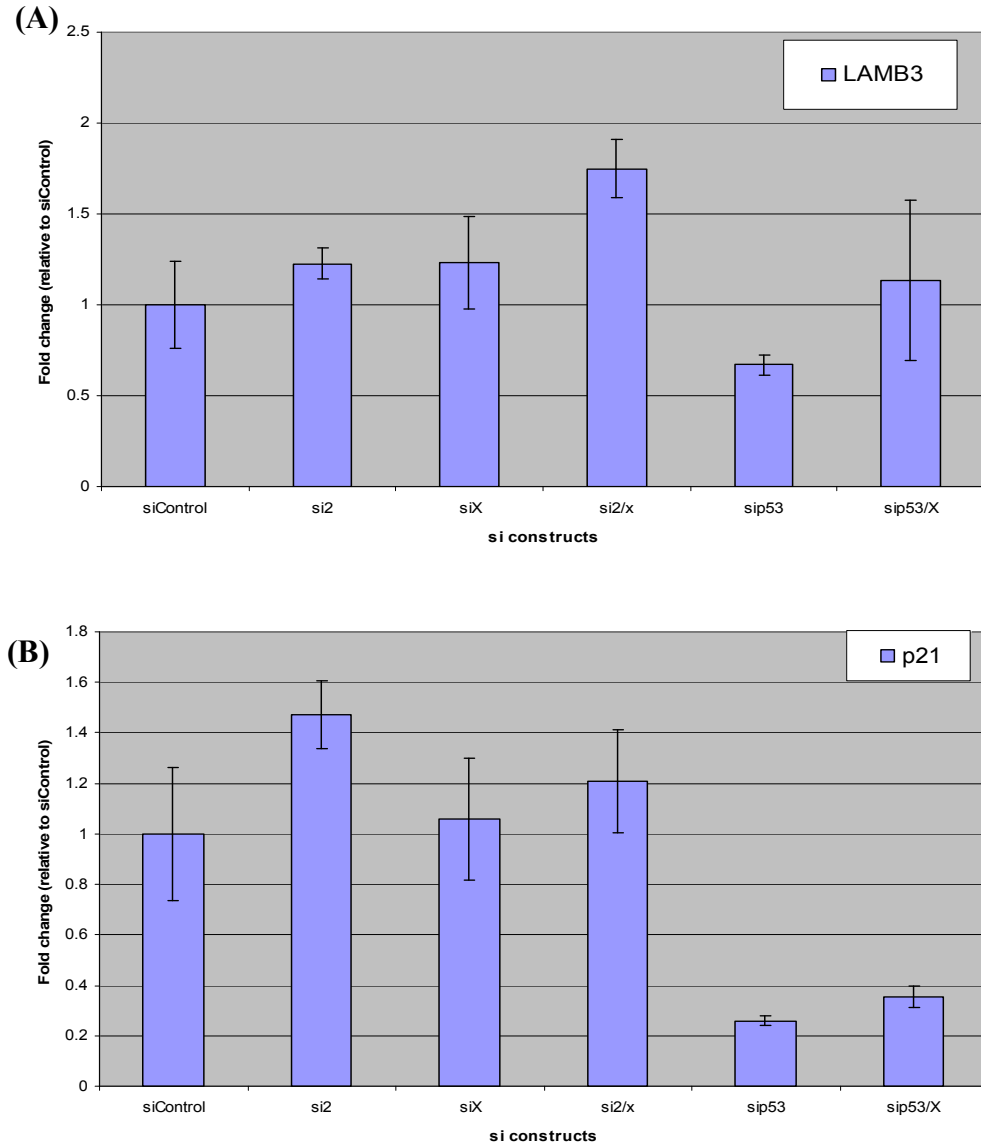


Figure 5: Knockdown of Hdm2/HdmX using siRNA treatment in 76N tert cells induces LAMB3 and p21. Triple transfection was carried out on 76N tert (Human immortalized mammary epithelial) cells for the knockdown of Hdm2, HdmX, Hdm2 and HdmX, p53 or HdmX and p53 as described in Materials and Methods. Twenty four hours after the last siRNA transfection RNA was extracted and reverse transcription was performed. Following this, Real-time PCR was carried out in triplicate to quantify the expression of **(A) LAMB3** and **(B) p21**. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to siControl, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars. This experiment was performed by Dr. Steven Berberich.

following the knockdown of p53. Interestingly the knockdown of HdmX resulted in greater p53 activation than the loss of Hdm2 (Dr. Kate Heminger). Thus, comparing the current validation experiment results of p21 and LAMB3 leads to the observation that both LAMB3 and p21 transcripts increase on the simultaneous knockdown of HdmX along with the combination of Hdm2 and HdmX while they decrease upon p53 knockdown, although the LAMB3 data was very modest when compared to p21 fold changes. Hence, LAMB3 does show several features of a putative p53 target gene. The percent knockdown of Hdm2, HdmX and p53 is given in Table 4.

Since the validation was performed in MCF7 cells which are tumor cells, the next task was to evaluate the expression of LAMB3 in MCF10A and 76N tert cell lines which are both immortalized but non-transformed human mammary epithelial cells. Triple transfection was performed on these cell lines using the following si constructs – siControl, siHdm2, siHdmX, siHdm2 + siHdmX, sip53 and siHdmX + sip53. RNA was extracted 24 hours after the last transfection followed by RT-qPCR (See Materials and Methods for details). When compared to siControl, in MCF10A, LAMB3 transcript levels showed a ~1.3 fold induction in siHdm2 transfected cells and ~1.5 fold in the combined transfection containing siHdm2 and siHdmX, but there was no significant decrease seen in sip53 transfected cells (Figure 4A). On the other hand, p21 transcript levels showed a ~2 fold induction upon siHdm2 and ~2.25 fold upon the combination of siHdm2 and siHdmX, a ~1.2 fold induction upon siHdmX. A 60% decrease in p21 expression was observed upon p53 knockdown and a 50% decrease with the combined knockdown of HdmX and p53 (Figure 4B). In 76N tert cells, compared to siControl,

LAMB3 transcript expression increased ~75% for the combination of siHdm2 and siHdmX, while no change was seen with Hdm2 knockdown. There was a statistically significant decrease observed in the LAMB3 transcript level for sip53 although oddly no change was seen in the combination knockout (siHdmX and sip53) (Figure 5A). As expected, siHdm2 and the combination of siHdm2 + siHdmX induced p21 mRNA levels ~40%, but there was almost a 70% reduction observed upon the knockdown of p53 and a 60% reduction for siHdmX + sip53 (Figure 5B). Although p21 and LAMB3 transcript levels did not show the expected increase with siHdmX in either of the cell lines, the percent knockdown of HdmX in both cell lines was ~60% (Table 4). The percent knockdown of Hdm2, HdmX and p53 in both MCF10A and 76N tert cell lines is given in Table 4.

LAMB3 transcript levels remain unaffected after p53 activation using siRNA towards Hdm2 and HdmX in U2OS cell line.

To evaluate the LAMB3 expression in a non-epithelial origin cell line U2OS (human osteosarcoma) cells were utilized in a siRNA knockdown experiment. Results showed that LAMB3 levels remained unaffected in all siRNA transfections relative to siControl (Figure 6A). On the contrary when compared to siControl, p21 transcript levels showed an induction of ~1.5 fold for siHdm2 and siHdmX, ~2 fold for siHdm2 + siHdmX, and a reduction of ~0.6 fold for sip53 and ~0.5 fold for siHdmX + sip53 (Figure 6B). This leads to the observation that LAMB3 expression relative to the si-knockdown maybe cell line specific.

Activation of p53 with DNA damage by Doxorubicin treatment in MCF7 cells leads to a reduction of LAMB3 transcript levels.

DNA damage is known to activate p53, wherein p53 is stabilized and functions as a transcription factor inducing genes involved in cell cycle arrest, DNA repair and apoptosis (Vousden & Lu, 2002). Doxorubicin is a DNA damaging agent which intercalates within chromosomal DNA and causes double-strand breaks. To check the response of LAMB3 transcripts following p53 activation through DNA damage, MCF7 cells were treated with increasing concentrations of doxorubicin. Twenty-four hours after doxorubicin treatment RNA was extracted, quantified and LAMB3 and p21 gene expression analyzed by RT-qPCR. p21 was used as a positive control (known p53 target gene) and relative expression normalized to GAPDH expression levels. LAMB3 levels did not increase in any of the doxorubicin treatment doses when compared with untreated MCF7 cells (Figure 7A). In contrast, p21 transcript levels displayed the expected dose dependent increase in expression from 0 through 0.25 $\mu\text{g/mL}$ of doxorubicin (Figure 7B). The decrease in p21 and LAMB3 gene expression observed at higher doxorubicin doses correlated with cell death. Thus, as predicted p21 transcript levels showed dose dependency upon doxorubicin damage in MCF7 cells but LAMB3 transcript levels were not induced by any dose of doxorubicin.

LAMB3 transcript levels show no response to DNA damage by Doxorubicin or Bleomycin in MCF10A cells.

To confirm that the negative result with LAMB3 expression upon DNA damage was not unique to tumor cell lines like MCF7 cells, I wanted to investigate the response

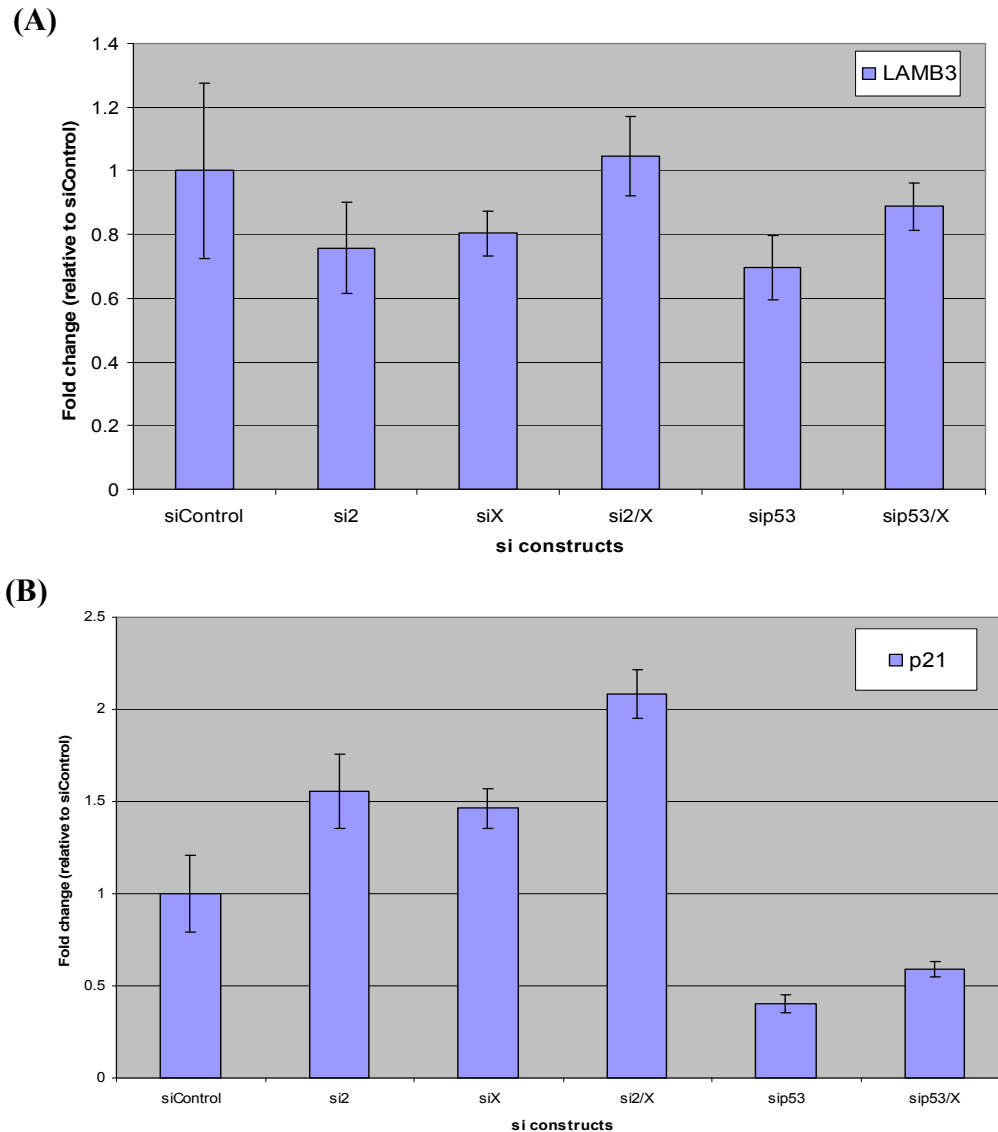


Figure 6: RNAi mediated knockdown of Hdm2/HdmX in U2OS cells shows no significant change in LAMB3 transcript levels but p21 transcript levels are induced. Triple transfection was carried out on U2OS (Human osteosarcoma) cells for the knockdown of Hdm2, HdmX, Hdm2 and HdmX, p53 or HdmX and p53 as described in Materials and Methods. Twenty four hours after the last siRNA transfection RNA was extracted and reverse transcription was performed. Following this, Real-time PCR was carried out in triplicate to quantify the expression of (A) LAMB3 and (B) p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to siControl, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

Cell line	Percent knockdown		
	Hdm2	HdmX	p53
MCF7	65%	64%	86%
MCF10A	40%	60%	67%
76N tert	N/A (error bars overlap)	62%	71%
U2OS	45%	50%	62%

Table 4: - Percent knockdown of Hdm2, HdmX and p53 in MCF7, MCF10A, 76N tert and U2OS cell lines. The above table lists the approximate percent knockdown of Hdm2, HdmX and p53 relative to siControl in MCF7, MCF10A, 76N tert and U2OS cell lines.

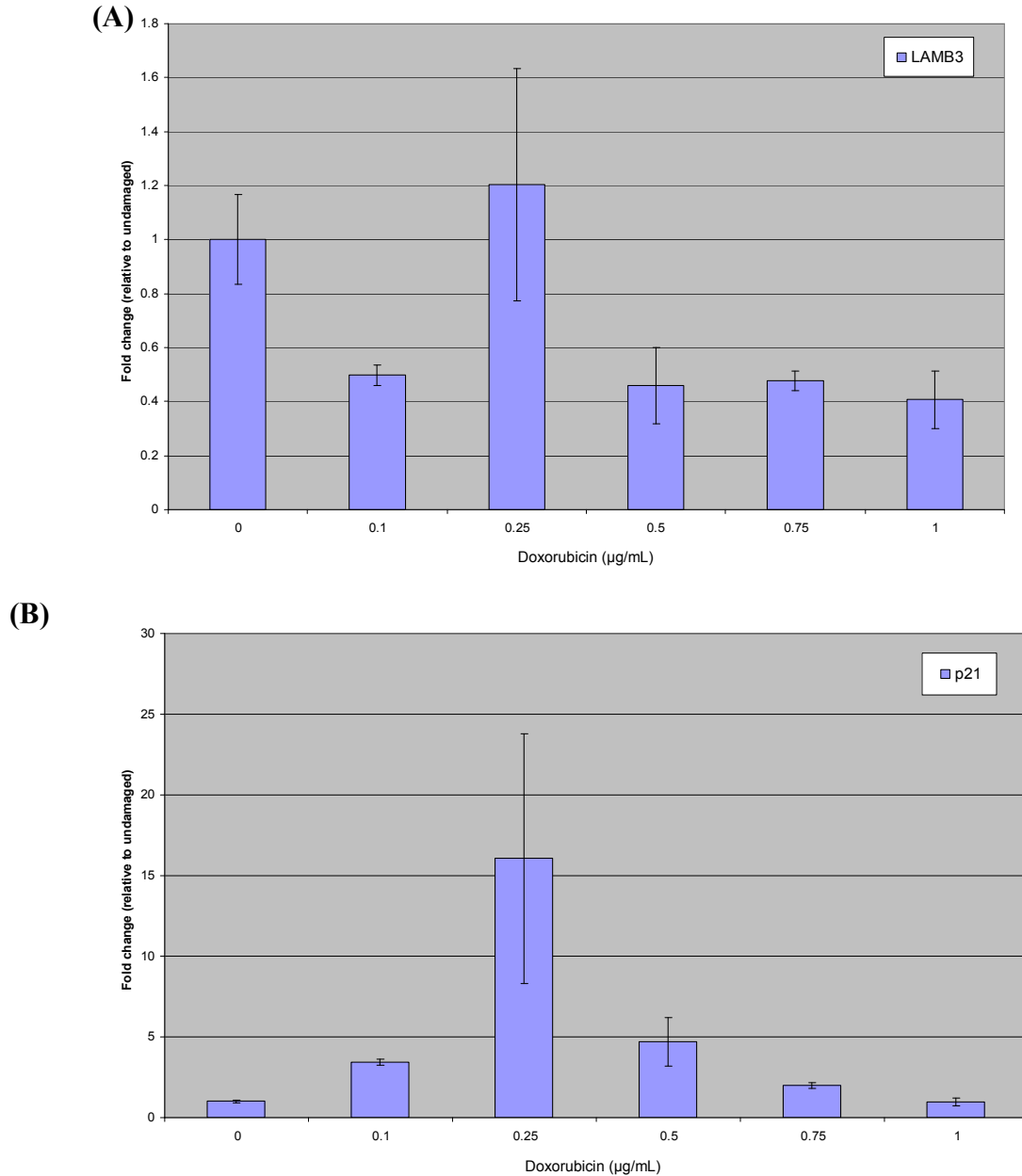
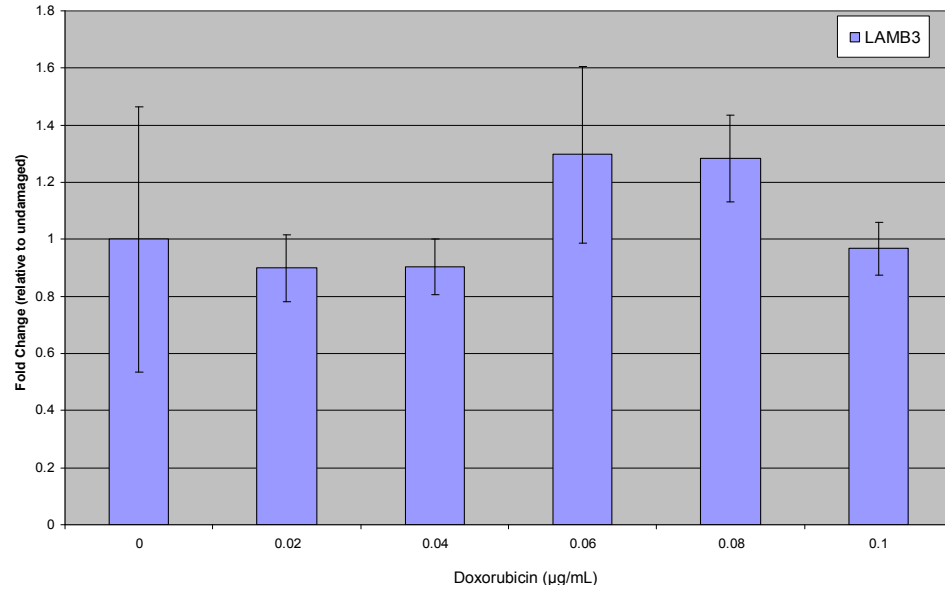


Figure 7: Transcript levels of LAMB3 are decreased upon doxorubicin treatment while those of p21 follow a dose dependent manner of expression in MCF7 cells. MCF7 cells were treated with increasing concentrations of the DNA-damaging agent Doxorubicin. Twenty-four hours after treatment with doxorubicin, RNA was extracted and Reverse transcription was carried out. Real-time PCR was then performed in triplicate to quantify the expression of **(A)** LAMB3 and **(B)** p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to undamaged, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

(A)



(B)

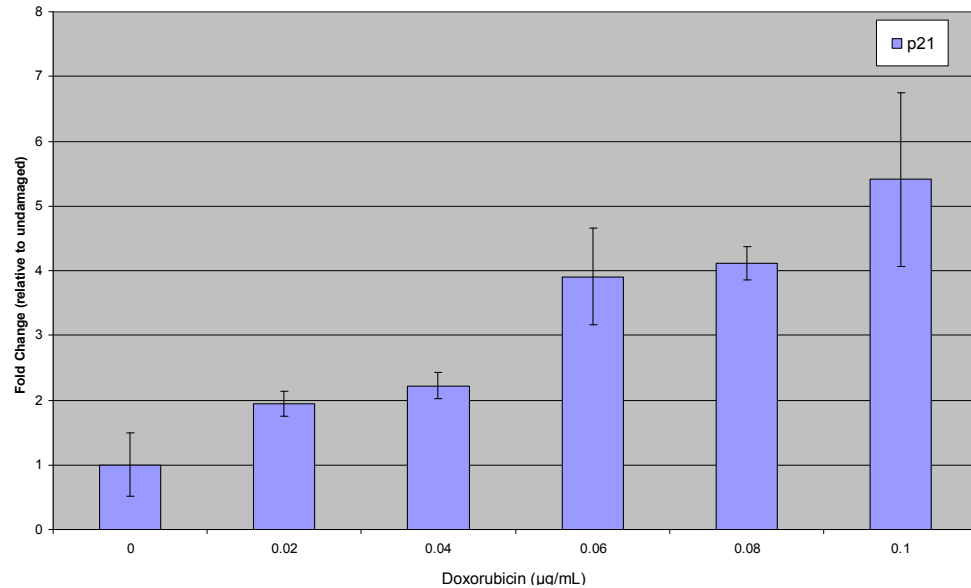


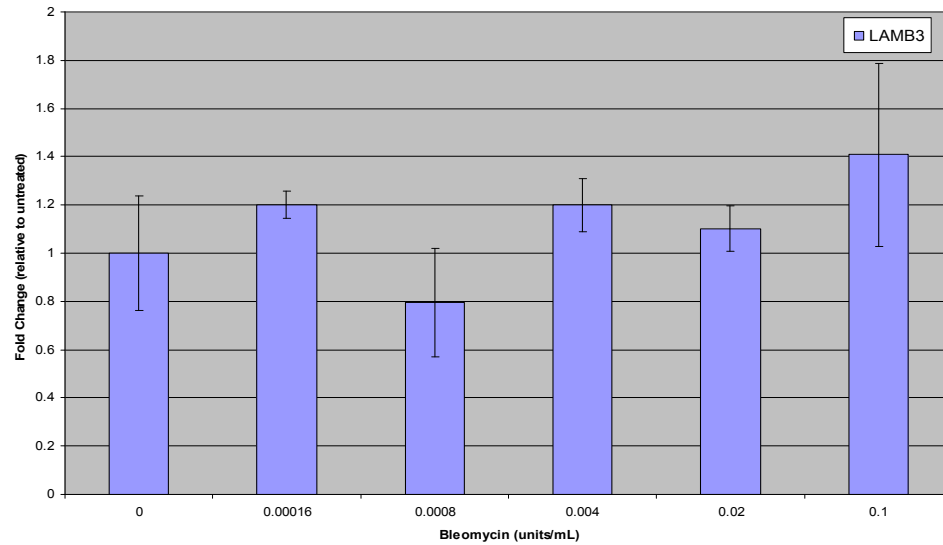
Figure 8: LAMB3 transcript levels are unaffected upon doxorubicin treatment while p21 transcripts show a dose dependent increase in MCF10A cells. MCF10A cells were treated with increasing concentrations of the DNA-damaging agent Doxorubicin. Twenty-four hours after treatment with doxorubicin, RNA was extracted and Reverse transcription was carried out. Real-time PCR was then performed in triplicate to quantify the expression of (A) LAMB3 and (B) p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to undamaged, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

of LAMB3 in a non-cancerous cell line like MCF10A which is non-transformed and immortalized. For this reason, MCF10A cells were treated with lower doses of doxorubicin since higher doses led to more cell death (data not shown). LAMB3 and p21 gene expression was examined by RT-qPCR. No change was observed in LAMB3 transcript levels while p21 transcript levels showed a dose dependent increase compared to the untreated sample (Figure 8A and 8B respectively). To examine whether the response of LAMB3 is DNA damage agent specific, I performed another experiment using a different DNA damaging agent namely Bleomycin in MCF10A cells. Here, MCF10A cells were treated with increasing doses of radiomimetic bleomycin which causes DNA strand breaks. RNA was used to assess LAMB3 and p21 gene expression by RT-qPCR. Analysis of the results showed a dose dependent increase in p21 transcript levels but LAMB3 transcripts remained unaltered relative to siControl (Figure 9A and 9B). Hence, it appears that activation of p53 by doxorubicin or bleomycin treatment does not affect LAMB3 expression in MCF10A cell line. Taken with the negative results in doxorubicin treated MCF7 cells it appears that LAMB3 transcripts are not impacted by p53 activation following genotoxic stresses. The observed increases in LAMB3 mRNA in the RNAi experiments may have been due to the activation of p53 in response to a non-genotoxic stress brought about by knockdown of its negative regulators (Hdm2 and HdmX).

Increase in LAMB3 transcripts upon DNA damage by Doxorubicin in HCT 116 +/+ p53 and HCT 116 -/- p53.

In contrast to the negative results observed in damaged epithelial cells, HCT 116 colon carcinoma cells did trigger a damage dependent increase in LAMB3 transcripts. This observation was derived from the experiment conducted wherein HCT 116 +/+ p53 and isogenic HCT 116 -/- p53 cell lines were treated with increasing concentrations of doxorubicin as the DNA damaging agent. One day following treatment of doxorubicin in both the cell lines, RNA was extracted and used to analyze gene expression levels of LAMB3 and p21. Analysis of RT-qPCR led to the observation that relative to the untreated sample, LAMB3 transcripts showed an increase of expression in two doses of doxorubicin induced DNA damage in both HCT 116 +/+ p53 (Figure 10A) and HCT 116 -/- p53 (Figure 11A) cell lines. p21 transcript levels increased in these same doses of doxorubicin in HCT 116 +/+ p53 cell line (Figure 10B) and HCT 116 -/- p53 cell line (Figure 11B). The decrease of LAMB3 and p21 transcripts in the two higher doses of doxorubicin in HCT 116 +/+ p53 cells and one dose in HCT 116 -/- p53 cells correlated with the observed cell death for the same doses. Also, the increase in LAMB3 transcripts after DNA damage by doxorubicin in HCT 116 -/- p53 cells led to the conclusion that LAMB3 can be induced in a p53 independent manner. Since, similar results were obtained with p21 we can deduce that in HCT 116 -/- cells, both LAMB3 and p21 maybe regulated by another transcription factor in a p53 – independent mechanism.

(A)



(B)

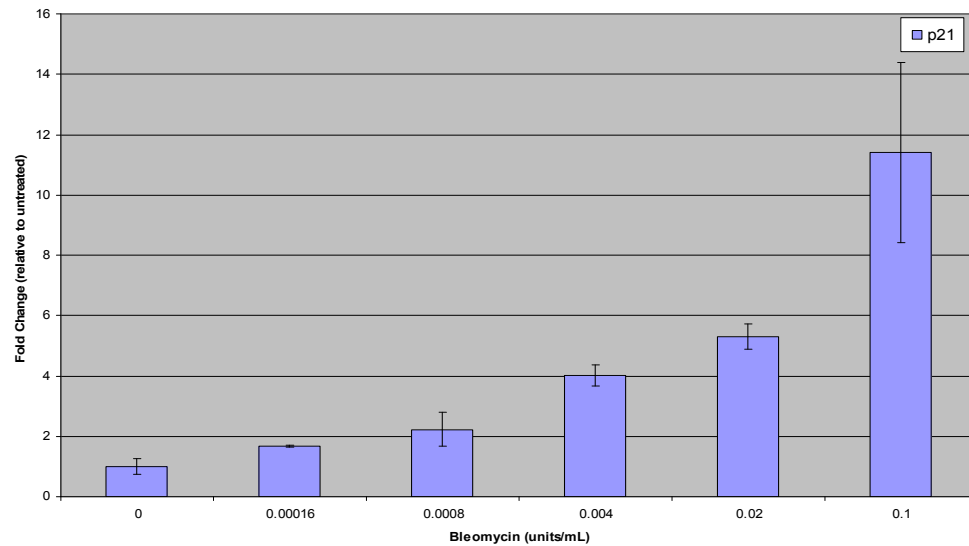


Figure 9: LAMB3 transcript levels are unaffected upon Bleomycin treatment but transcripts of p21 show a dose dependent increase of expression in MCF10A cells. MCF10A cells were treated with increasing concentrations of the DNA-damaging agent bleomycin. Twenty-four hours after treatment with bleomycin, RNA was extracted and reverse transcription was carried out. Real-time PCR was then performed in triplicate to quantify the expression of (A) LAMB3 and (B) p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to undamaged, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

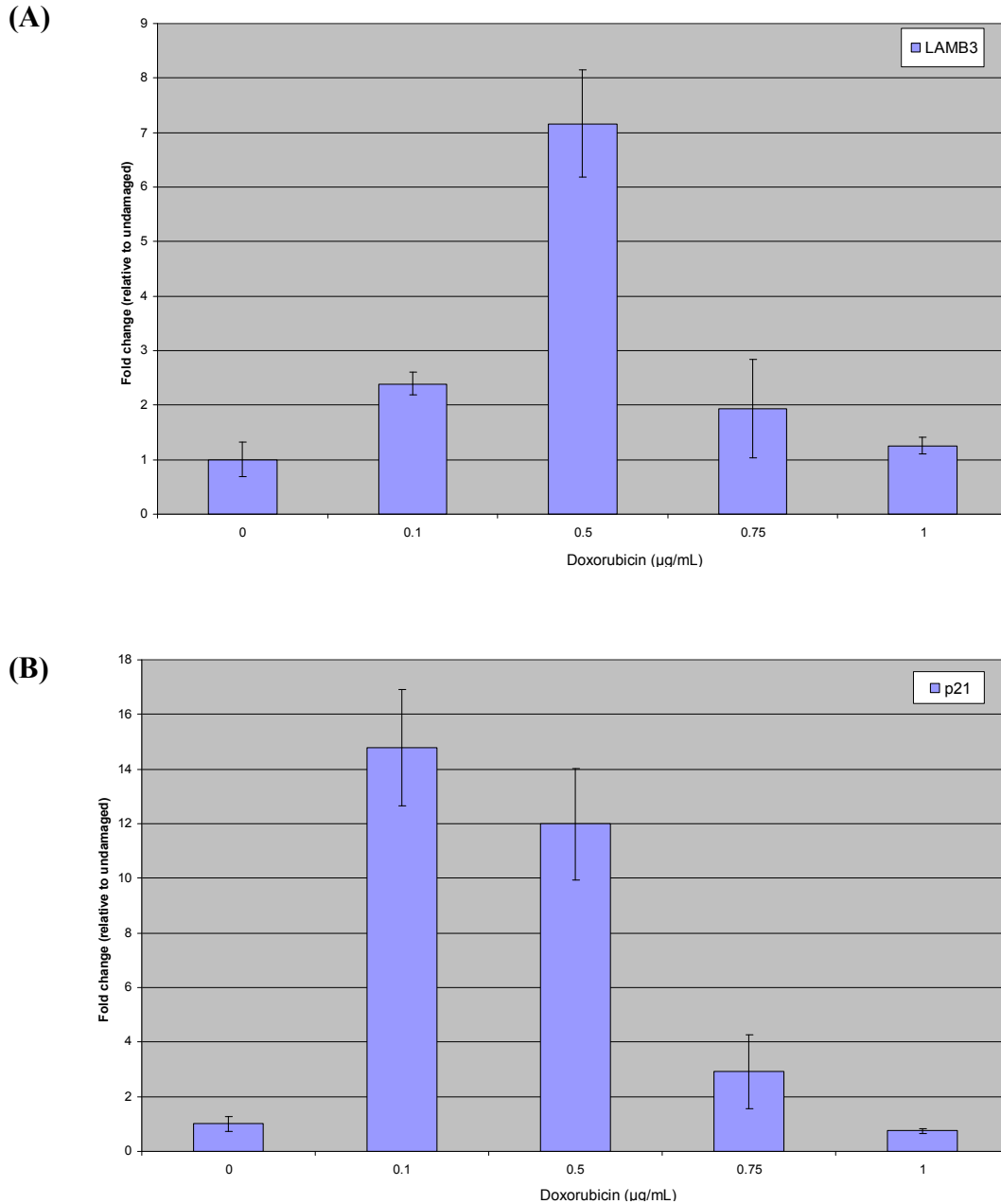
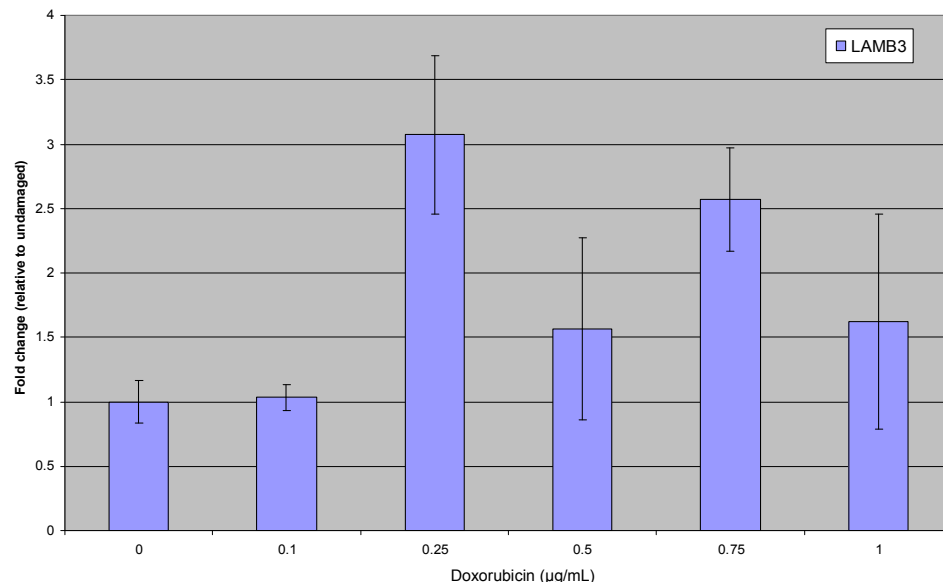


Figure 10: LAMB3 transcript levels show a dose dependent increase while p21 transcript levels increase upon doxorubicin treatment in HCT 116 +/+ p53 cells. HCT 116 p53 +/+ cells were treated with increasing concentrations of the DNA-damaging agent Doxorubicin. Twenty-four hours after treatment with doxorubicin, RNA was extracted and Reverse transcription was carried out. Real-time PCR was then performed in triplicate to quantify the expression of (A) LAMB3 and (B) p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to undamaged, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

(A)



(B)

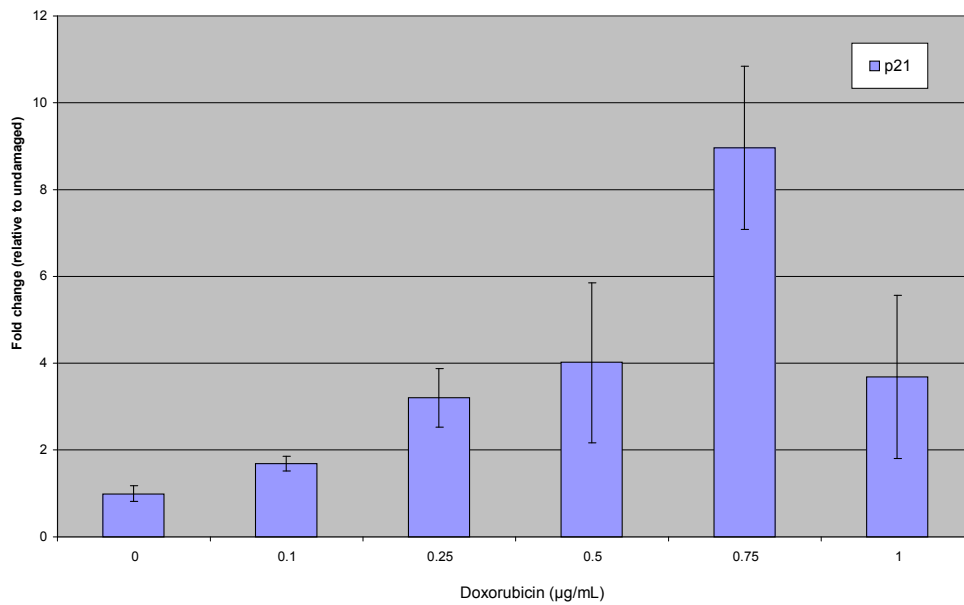


Figure 11: LAMB3 transcript levels are increased upon doxorubicin treatment and p21 shows a dose dependent increased pattern of expression in HCT 116 ^{-/-} p53 cells. HCT 116 p53^{-/-} cells were treated with increasing concentrations of the DNA-damaging agent Doxorubicin. Twenty-four hours after treatment with doxorubicin, RNA was extracted and Reverse transcription was carried out. Real-time PCR was then performed in triplicate to quantify the expression of (A) LAMB3 and (B) p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to undamaged, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

CELL LINE	LAMB3 transcript level response to Doxorubicin treatment (0 – 1 µg/mL)
MCF7	Decrease
MCF10A	Unaffected
HCT 116 +/+ p53	Increase
HCT 116 -/- p53	Increase

Table 5: - LAMB3 transcript level response to DNA-damage by Doxorubicin. The table shows LAMB3 response in various cell lines to DNA-damage induced by Doxorubicin.

Table 5 gives a summary of the effect of doxorubicin induced DNA damage on LAMB3 transcript levels in MCF7, MCF10A, HCT 116 +/+ p53 and HCT 116 -/- p53 cells.

Transcripts of LAMB3 change over 0, 6, 12 and 24 hours upon DNA damage by 0.25 µg/mL dose of Doxorubicin in MCF7 and HCT 116 +/+ p53 cells while they remain constant in HCT 116 -/- cells.

In the DNA damage experiments I had evaluated LAMB3 transcript expression at 24 hours. It could be possible that 24 hours post damage was not the optimal time to monitor LAMB3 transcript levels. Thus, to determine whether 24 hours after treatment was the optimal time to examine changes in expression levels of LAMB3; MCF7, HCT 116 +/+ p53 and HCT 116 -/- p53 cell lines were treated with 0.25 µg/mL concentration of doxorubicin. RNA was extracted before and 6, 12 and 24 hours after exposure to doxorubicin. Reverse transcription was performed followed by RT-qPCR. Results showed that 0, 6, 12 and 24 hours after treatment with 0.25 µg/mL of doxorubicin the LAMB3 transcript decreased in MCF7 cells (Figure 12A), increased in HCT 116 +/+ p53 cells (Figure 12B) and remained almost constant in HCT 116 -/- p53 cells when compared to undamaged (Figure 12C). On the other hand, p21 transcript levels increased over 0-24 hours after 0.25 µg/mL in MCF7 (Figure 12D), HCT 116 +/+ p53 (Figure 12E) and HCT 116 -/- p53 (Figure 12F). Taken together the data shows that 24 hours was the optimal time to analyze LAMB3 response to DNA damage.

Summarizing the DNA damage results it appears that LAMB3 expression levels are unaffected in the two epithelial cell lines, MCF7 and MCF10A upon treatment with doxorubicin at 24 hours while there is an increase in both the HCT 116 colon cancer cell lines under the same condition. DNA damage induces a genotoxic stress which activates p53 leading to a transactivation of its target genes (eg: - p21, Fas, GADD45 etc.) (Vousden & Lu, 2002). The overall observation from the DNA damaging experiments is that upon DNA damage if LAMB3 is transactivated by p53, then the transactivation is cell line specific. In addition, the increase in LAMB3 transcript levels in the HCT 116 -/- p53 cell line indicates that the regulation of LAMB3 can also take place in a p53 independent manner.

Over expression of p53, p63 γ and p73 α in H1299, SaOS2, HCT 116 -/- p53 and U2OS cells does not show change in LAMB3 transcript levels.

The observation that upon DNA damage, LAMB3 and p21 mRNA levels increased in a p53 independent manner in HCT 116 -/- p53 cell lines (Figure 11) led to the conclusion that there is a possibility of another transcriptional factor regulating both p21 and LAMB3 gene expression in the absence of p53. Several groups have reported that p63 and p73 (p53 family members) over expression up-regulates p21 transcription (Jost, Marin, & Kaelin, 1997; Osada et al., 1998). p73 has not only been shown to differentially regulate cellular p53 target genes but also activates p53 regulated genes following doxorubicin treatment of a p53-deficient breast cancer cell line (Vayssade et al., 2005; Zhu, Jiang, Zhou, & Chen, 1998). In view of the above mentioned studies I wanted to determine whether the p53 family members can transactivate LAMB3 in the

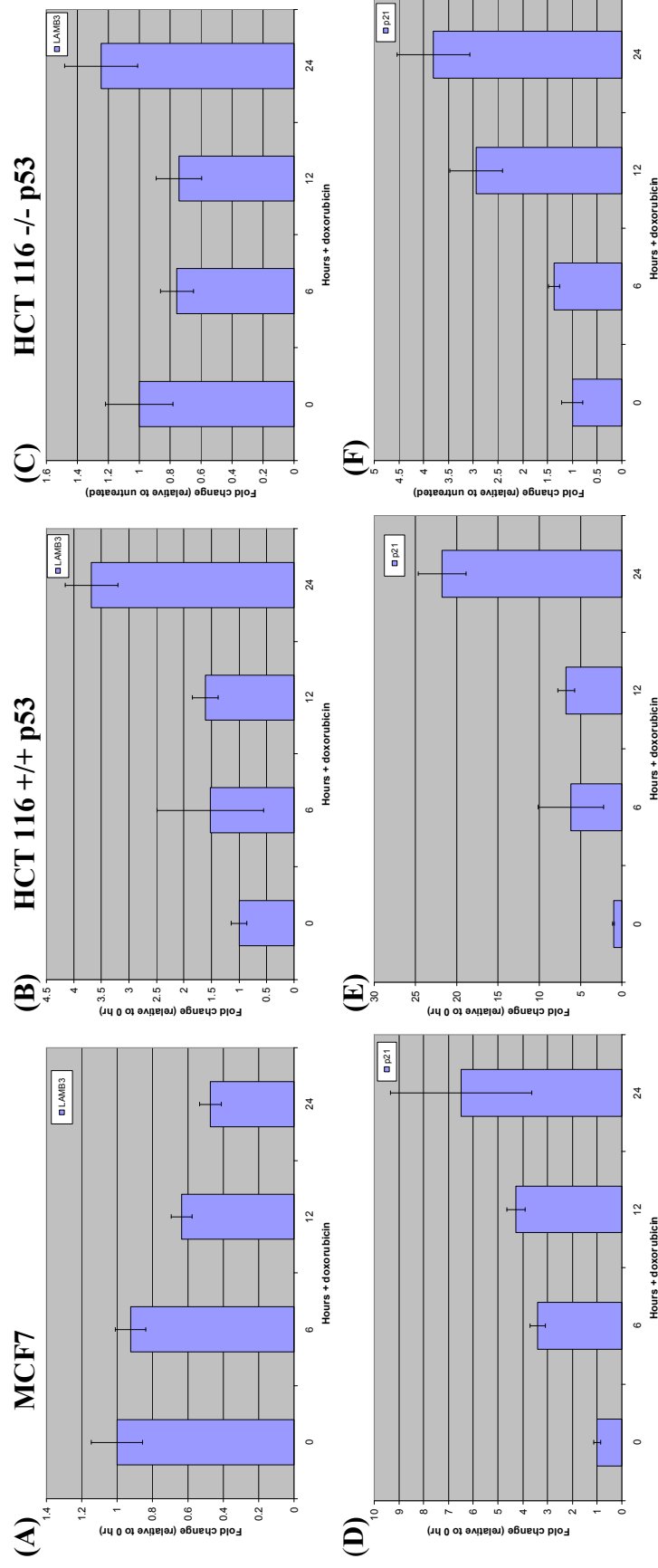


Figure 12: Changes in transcript levels of LAMB3 and p21 over 0-24 hours upon DNA-damage by doxorubicin treatment of 0.25 $\mu\text{g/mL}$. (A),(D) MCF7 (B),(E) HCT 116 +/- p53 (C),(F) HCT 116 -/- p53 cells were treated with 0.25 $\mu\text{g/mL}$ concentration of the DNA-damaging agent Doxorubicin. RNA was extracted before treatment and at 6, 12 and 24 hrs after treatment with doxorubicin followed by Reverse transcription. Real-time PCR was then performed in triplicate to quantify the expression of (A) (B)(C) LAMB3 and (D)(E)(F) p21. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to undamaged, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

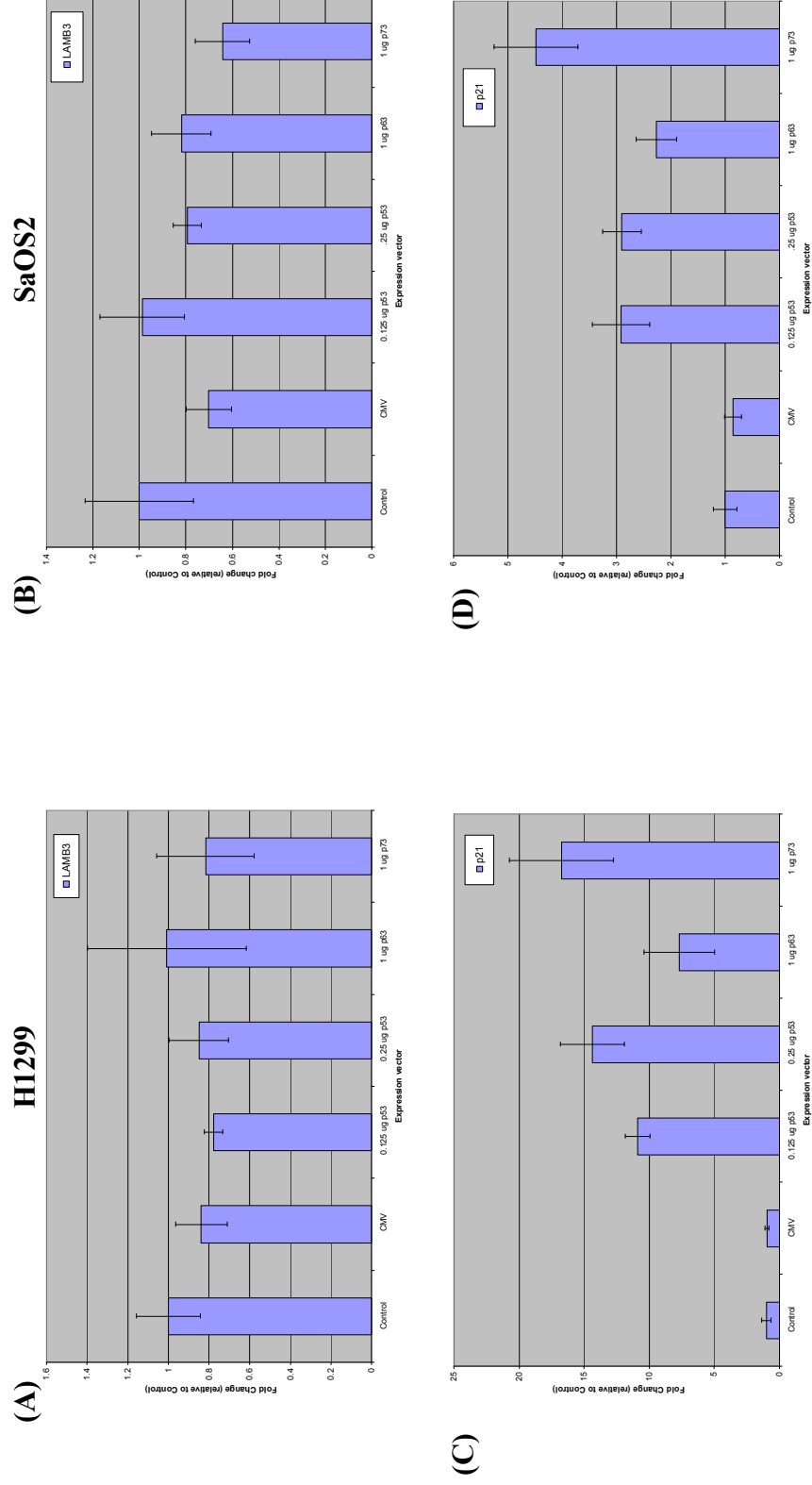


Figure 13: Over expression of p53, p63 γ and p73 α in H1299 and SaOS2 cells. (A, C) H1299 and (B, D) SaOS2 cells were transfected with 0.125 μ g and 0.25 μ g of p53, 1 μ g each of p63 and p73 as given in Materials and Methods. Twenty-four hours after transfection RNA was extracted and reverse transcription was carried out. Real-time PCR was then performed in triplicate to quantify the expression of (A, B) LAMB3 which was unaffected and (C, D) p21 whose levels increased. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to Control (non-transfected), using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars. This experiment was performed by Kevin Kelley.

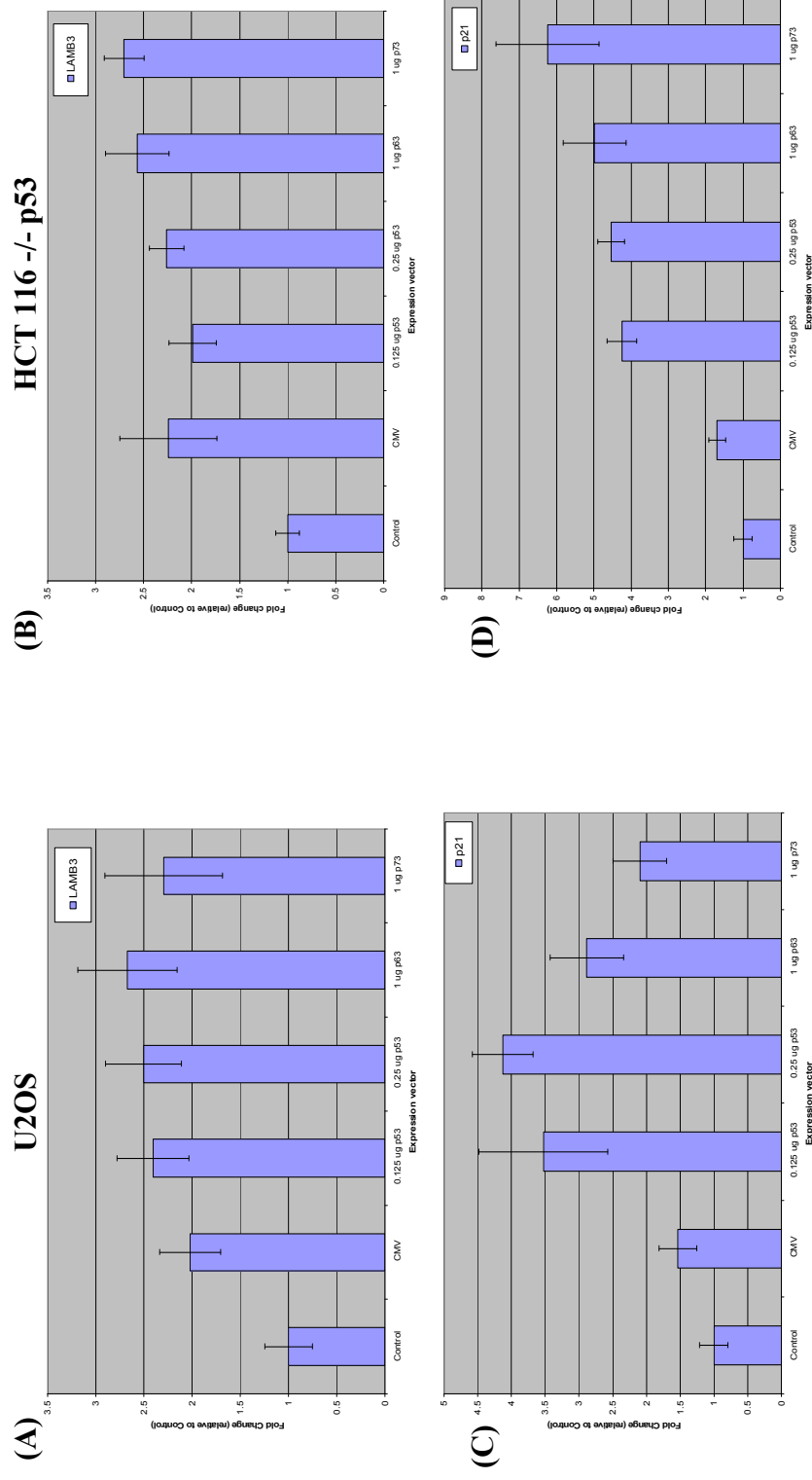


Figure 14: Over expression of p53, p63 γ and p73 α in U2OS and HCT 116 -/- p53 cells. (A, C) U2OS and (B, D) HCT 116 -/- p53 cells were transfected with 0.125 μ g and 0.25 μ g of p53, 1 μ g each of p63 and p73 as given in Materials and Methods. Twenty-four hours after transfection RNA was extracted and reverse transcription was carried out. Real-time PCR was then performed in triplicate to quantify the expression of (A, B) LAMB3 which was unaffected and (C, D) p21 whose levels increased. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to Control (non-transfected), using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars. This experiment was performed by Kevin Kelly.

absence of p53. Thus, p53, p63 γ and p73 α were overexpressed in H1299, HCT 116 -/- p53, SaOS2 cells (p53 null) and U2OS cells (with p53). LAMB3 transcripts remained unaffected with overexpression of p53, p63 γ and p73 α in H1299, SaOS2, U2OS and HCT 116 -/- p53 (Figure 13A, 13B, 14A and 14B respectively). On the contrary, p21 transcript levels increase in all the above mentioned cell lines (irrespective of p53 status) upon over expression of p53, p63 γ and p73 α (Figure 13C, 13D, 14C and 14D respectively). Thus, in the cell lines studied above overexpression of p53, p63 γ and p73 α does not regulate LAMB3 transcript levels.

Endogenous LAMB3, LAMA3 and LAMC2 levels are elevated in non-transformed, immortalized mammary epithelial cell lines.

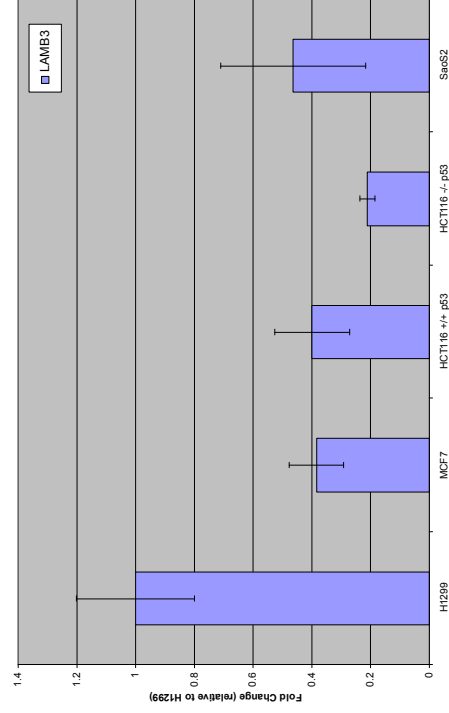
To compare endogenous LAMB3 levels, the cell lines H1299, MCF7, HCT 116 +/- p53, HCT 116 -/- p53, SaOS2, MCF10A and 76N tert were utilized. RT-qPCR was used to determine the basal expression pattern of LAMB3, LAMA3 and LAMC2 transcripts – which are translated to form the three chains of laminin-5. When compared to H1299 cells (p53 null), endogenous LAMB3 mRNA levels were lower in all cell lines except MCF10A which showed a ~37 fold and 76N tert which had ~29 fold higher amount of endogenous LAMB3 mRNA levels (Figure 15A and 15B). The point to note is that LAMB3 gene expression is higher in the immortalized but non-transformed mammary epithelial cell lines of MCF10A and 76N tert as compared to tumor cell lines. Thus, basal LAMB3 expression levels differ in all the cell lines incorporated in this experiment.

Laminin-5 consists of three polypeptide chains LAMA3, LAMB3 and LAMC2. Thus, I tested endogenous levels of LAMA3 and LAMC2 in various cell lines. Table 6 gives the RQ values for LAMA3 and LAMC2 transcripts in the cell lines. When compared to H1299 all cell lines except SaOS2 had higher transcript levels of LAMA3. In SaOS2, mRNA levels of LAMA3 were almost similar to that of H1299. On the other hand, all cell lines except MCF10A and 76N tert had lower levels of LAMC2 when compared to H1299. Hence, as in the case of LAMB3 both immortalized but non-transformed mammary epithelial cell lines (MCF10A and 76N tert) also had the highest levels of LAMA3 and LAMC2 (Table 6).

LAMB3 protein is absent in MCF7 cells while it is present in H1299, HCT 116 -/- p53, SaOS2 and MCF10A cell lines.

In a study conducted by Akutsu et al., (Akutsu, Amano, & Nishiyama, 2005) it was observed that in keratinocytes the absolute mRNA levels produced from the laminin-5 genes do not determine the translated protein levels. Considering this report and the observation from an earlier experiment conducted to look at endogenous LAMB3 mRNA levels in various cell lines (Figure 15), I used western blotting technique to look at whether endogenous LAMB3 transcript levels co-related with protein expression (See Materials and Methods for details). MCF7, H1299, SaOS2, MCF10A and HCT 116 -/- p53 cell lines were used for this experiment. LAMB3 protein was observed in H1299 and HCT 116 -/- p53 cell lines (Figure 16). It was unexpected to observe high amounts of LAMB3 protein levels in H1299 and HCT 116 -/- p53 cell lines which are devoid of p53. But, this data supports the observation that LAMB3 expression is independent of p53.

(A)



(B)

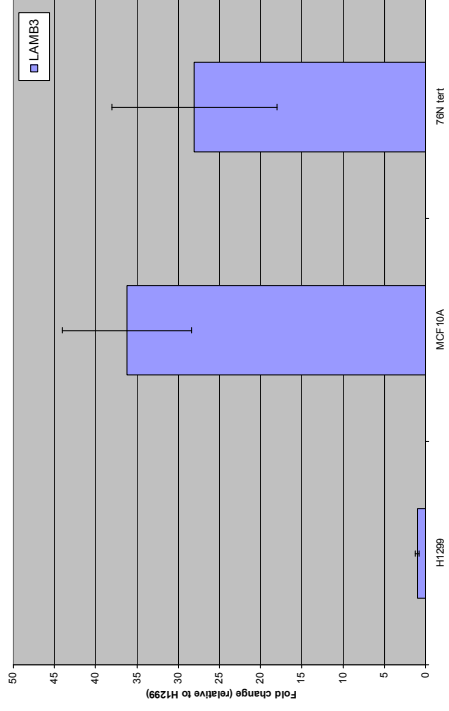


Figure 15: Endogenous LAMB3 mRNA levels are elevated in non-transformed, immortalized mammary epithelial cells. The cell lines (A) H1299, MCF7, HCT 116 p53 $+/+$, HCT 116 p53 $-/-$, SaOS2, (B) MCF10A and 76N test were cultured. Twenty four hours later RNA was extracted and reverse transcription was performed. Following this, Real-time PCR was carried out in triplicate to quantify the expression of LAMB3. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to H1299, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

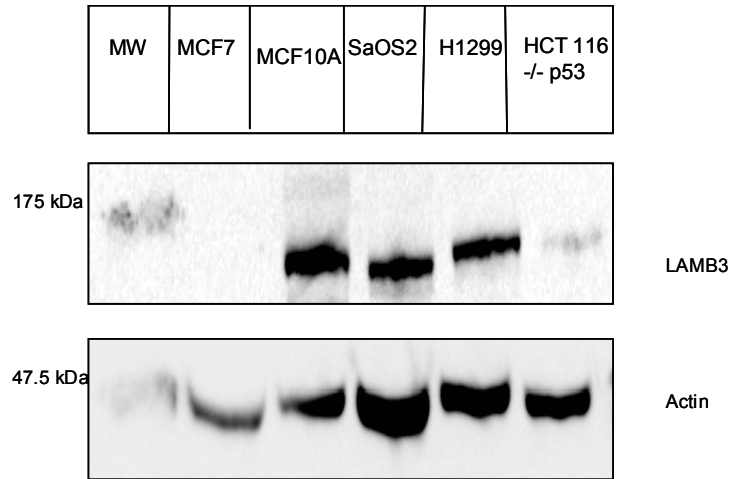


Figure 16: - LAMB3 protein expression absent in MCF7 cells but present in H1299, HCT 116 -/- p53, SaOS2 and MCF10A cells. The above mentioned cells were cultured, protein extracted and quantified by Bradford assay. (A) 200 µg of protein was loaded onto a 10% SDS-PAGE gel. Actin was used as a loading control.

	LAMA3			LAMC2		
	RQ	RQ min	RQ max	RQ	RQ min	RQ max
H1299	1	0.78	1.28	1	0.88	1.14
MCF7	15.79	13.38	18.64	0.23	0.19	0.29
HCT 116 +/+ p53	11.78	9.62	14.43	0.50	0.39	0.64
HCT 116 -/- p53	7.84	6.86	8.96	0.39	0.32	0.47
SaOS2	1.13	0.79	1.63	0.53	0.39	0.72
MCF10A	3843.36	3207.57	4605.18	87.52	72.42	105.77
76N tert	2015.12	1508.41	2692.05	41.70	32.04	54.27

Table 6: Endogenous mRNA levels of LAMA3 and LAMC2 are elevated in non-transformed, immortalized mammary epithelial cells. The cell lines H1299, MCF7, HCT 116 p53 +/+, HCT 116 p53 -/-, SaOS2, MCF10A and 76N tert were cultured. Twenty four hours later RNA was extracted and reverse transcription was performed. Following this, Real-time PCR was carried out in triplicate to quantify the expression of LAMA3 and LAMC2 relative to H1299, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value.

Surprisingly, MCF7 cells did not show any LAMB3 protein expression (Figure 16). In addition, SaOS2 (p53 null) and MCF10A (immortalized but non-transformed) cells were analyzed for LAMB3 protein expression levels (Figure 16). LAMB3 protein was also seen in SaOS2 and MCF10A (immortalized but non-transformed) cells.

Two mRNA isoforms of LAMB3 exist and have differential transcriptional expression.

Hao et al. (Hao, McDaniel, Weyer, Barrera, & Nagle, 2002) identified two mRNA isoforms of LAMB3 (referred to as β 3A and β 3B). β 3B was expressed in all cell lines examined but does not result in the production of LAMB3 protein alone. β 3B protein expression requires the expression of β 3A mRNA isoform in the cell lines studied by Hao and group (Hao et al., 2002). The MCF7 cell line was shown to be devoid of the protein coding β 3A isoform (Hao et al., 2002). This maybe the reason why we do not see LAMB3 protein in MCF7 cells (Figure 16). To evaluate and re-confirm Hao et al's results a series of cell lines were screened with primers specific for the β 3A and β 3B and also common to both the isoforms (β 3A- β 3B). RNA was extracted after twenty-four hours and treated with DNase. The DNase treated RNA was used in SYBR[®] Green PCR assay which was employed to quantify the expression levels of each of the mRNA isoforms of LAMB3 along with the combined expression of both the isoforms. The primers for the SYBR[®] Green PCR assay were made based on the information about the two LAMB3 mRNA isoforms listed in the UCSC Genome browser (See Materials and Methods for details). Figure 17 gives the diagrammatic representation of both the

isoforms of LAMB3 and Figure 18 gives the diagrammatic representation of the positions of the primer sets for the specific LAMB3 mRNA isoform and the combination.

The SYBR[®] Green PCR assay performed above resulted in differential expression of both β 3A and β 3B in the cell lines used. It was observed that both the mRNA isoforms of LAMB3 had the highest expression in the MCF10A and the 76N tert non-transformed but immortalized mammary epithelial cell line (Figure 19A and 19B) when compared to H1299 cells. Although, LAMB3 protein expression was higher in H1299 compared to the HCT 116 -/- p53 cell line (Figure 16) the expression level of the β 3A isoform was almost similar in both cell lines for this experiment (Figure 19A). Surprisingly, the β 3A isoform was observed in the MCF7 cells which were supposed to be lacking the β 3A isoform according to Hao et al. (Hao et al., 2002) (Figure 19A). Combined expression levels of both isoforms evaluated by using the primer common to both (β 3A- β 3B) showed an increase in the overall LAMB3 transcript expression (data not shown). Since MCF7 cell line showed the presence of the β 3A isoform, I performed another SYBR[®] Green Assay wherein I checked for genomic DNA contamination by not adding reverse transcriptase while making cDNA. In spite of no genomic contamination, the β 3A isoform was detected in MCF7 (data not shown).

Both LAMB3 mRNA isoforms show similar expression pattern in MCF7, HCT 116 +/- p53 and HCT 116 -/- p53 upon DNA damage by Doxorubicin.

To evaluate the level of expression of each isoform of LAMB3 contributing to the result for the overall LAMB3 transcript, RNA from previous DNA damage experiments

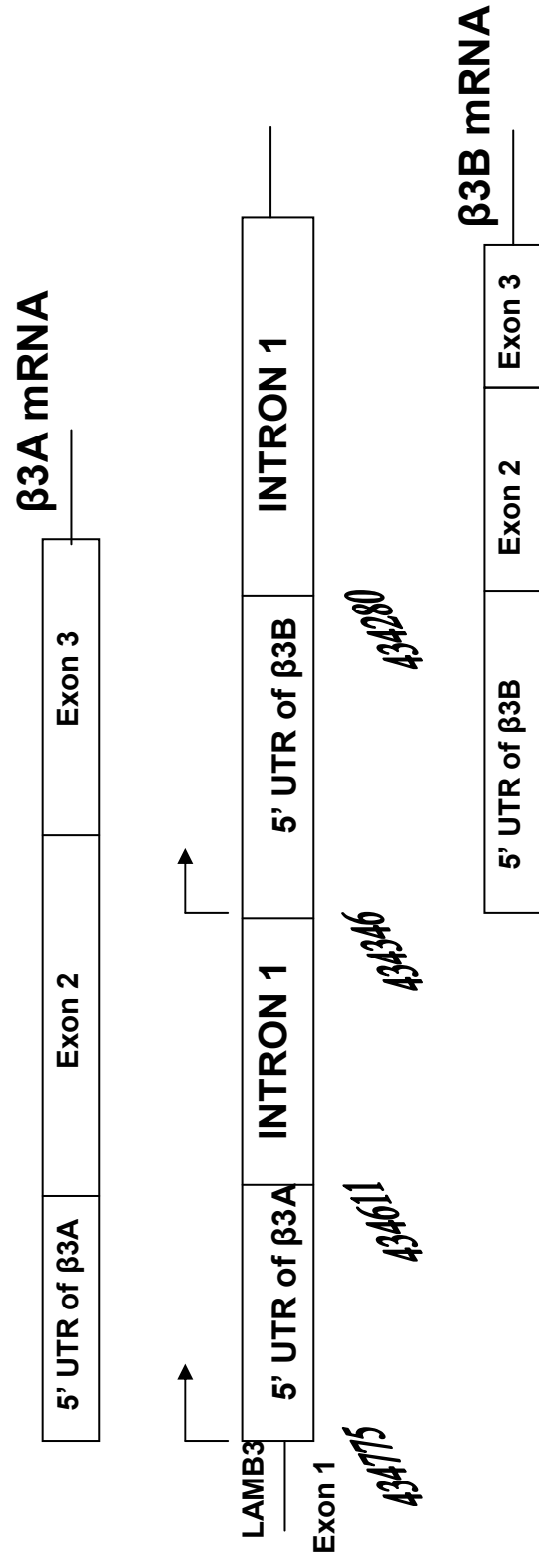


Figure 17: - Diagrammatic representation of two LAMB3 mRNA isoforms. The figure shows a diagrammatic representation of the two LAMB3 mRNA isoforms β3A and β3B. Both the isoforms have different transcriptional start sites.

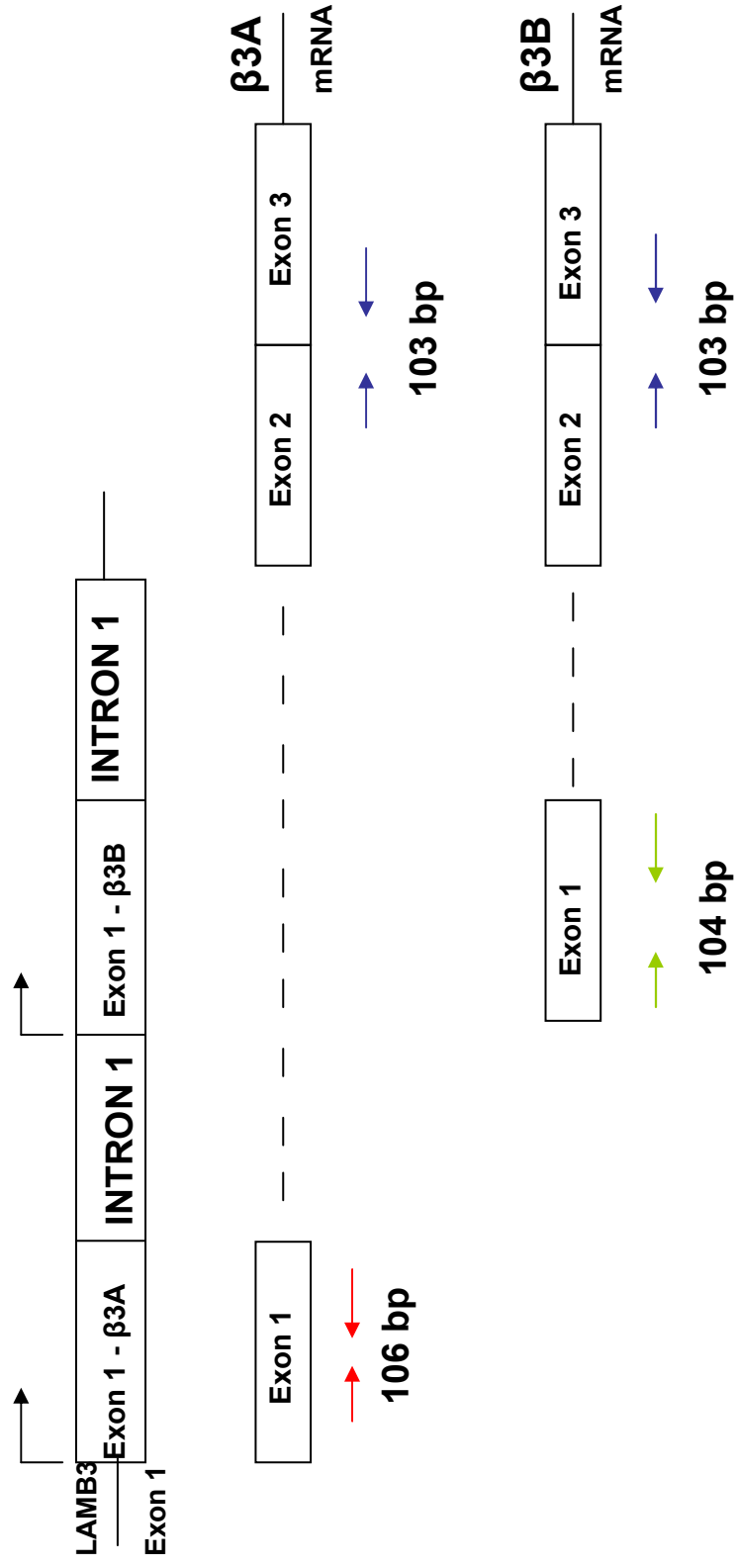


Figure 18: - Diagrammatic representation of primers designed for SYBR® Green PCR Assay of LAMB3 mRNA isoforms. For specific amplification of the different isoforms of LAMB3 namely β3A and β3B and the combination, primers (color coded arrows) were generated using the UCSC genome browser and obtained from Integrated DNA technologies. The primer spanning site and the product size are as mentioned according to color code in the diagram: - 1) **Red**: - primer spanning the first exon and specific to the β3A isoform with a product size of 106 bp. 2) **Green**: - primer spanning the second and third exon of both β3A and β3B isoform with a product size of 104 bp. 3) **Blue**: - primer spanning the first exon and specific to the β3B isoform with a product size of 103 bp. The primer sequences and other details are mentioned in Materials and Methods.

using doxorubicin for MCF7, HCT 116 +/+ p53 and HCT 116 -/- p53 (Figure 7, 10 and 11 respectively) was treated with DNase. DNase treated RNA was used to assess expression levels of both the LAMB3 isoforms and the combination by the SYBR[®] Green PCR assay. It was observed that when compared to the untreated cells, with increasing doses of doxorubicin both β 3A and β 3B show a statistically significant decrease in MCF7 cells (Figure 20A), show a dose dependent increase of expression in HCT 116 +/+ p53 cells (Figure 20B) and show a statistically significant increase in HCT 116 -/- p53 cells (Figure 20C). Using the primer common to both isoforms (β 3A- β 3B) the expression level of the combination was determined. This also displayed the same result as individually for each cell line (Table 8).

Table 7 summarizes the similar response of both mRNA isoforms in MCF7, HCT 116 +/+ p53 and HCT 116 -/- p53 upon doxorubicin induced DNA damage.

LAMB3 transcripts mimic p21 transcript level changes after Serum deprivation in MCF7 cells.

To prevent the emergence and propagation of cancer-prone cells, p53 is known to orchestrate either a pro-apoptotic transcription program or a growth arrest (Oren, 2003). Mosner and Deppert (Mosner & Deppert, 1994) observed that upon serum starvation p53 biosynthesis was lowered in the G₀ and G₁ phase of the cell-cycle (Mosner & Deppert, 1994; Mosner et al., 1995; Reisman & Loging, 1998). On account of these reports, I was interested to analyze LAMB3 expression in response to cell-cycle arrest. Thus, MCF7 cells were serum deprived for 24 and 48 hours. RNA was extracted at both time points

and after prior to serum deprivation (Asynchronous). The gene expression levels of LAMB3, p21, CCNA2 and p53 were determined using this RNA by RT-qPCR. At the same point of time, Flow Cytometry analysis was carried on the MCF7 cell samples for each time point (24 and 48 hours) to determine amount of cell cycle arrest and apoptosis (See Materials and Methods for details). The results obtained from RT-qPCR indicate that transcript levels of LAMB3 mimic that of p21 (known p53 target gene) i.e. the levels of LAMB3 and p21 transcripts reduce after serum deprivation at 24 and 48 hours (Figure 21A and 21B). CCNA2 which is used as a marker of cell cycle progression also shows a decrease in the transcript levels (Figure 21C). As expected, p53 reduces transcriptionally upon serum deprivation in MCF7 cells (Figure 21D). After evaluating the results it appears that upon serum starvation reduced LAMB3 transcripts correspond to a reduction in p53 levels. Flow Cytometry analysis shows that both 24 and 48 hr serum deprivation cause an increase in the G1 phase cells and a decrease in the S phase cells confirming the occurrence of a cell cycle arrest while the observed increase in sub-G1 indicates occurrence of apoptosis in cells (Figures 22A, 22B, 22C and 22D).

LAMB3 transcripts increase with the onset of Oncogenic ras induced Senescence

Cellular senescence is known as the phenomenon where normal diploid differentiated cells lose the ability to divide further and then the cells are known to senesce. Serrano et al. (Serrano, Lin, McCurrach, Beach, & Lowe, 1997) showed that oncogenic ras provokes premature senescence wherein accumulation of p53 and p16INK4a levels is seen. As a last resort, to examine LAMB3 response to stress, IMR90 cells were transduced with LEGFP and LRas constructs (See Materials and Methods for

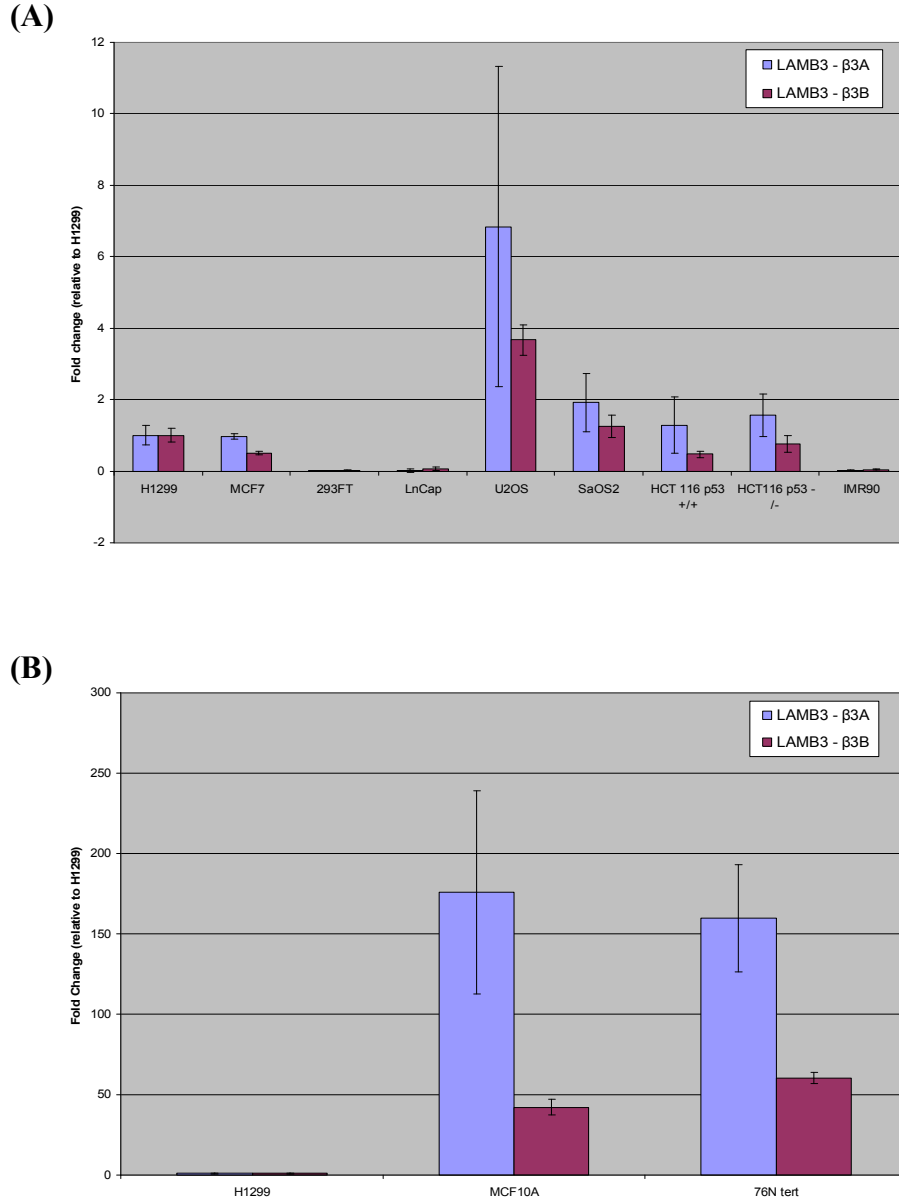


Figure 19: Endogenous levels of both LAMB3 mRNA isoforms β 3A and β 3B are elevated in non-transformed, immortalized mammary epithelial cells. (A) MCF7, 293FT, H1299, LnCap, U2OS, SaOS2, HCT 116 +/+ p53, HCT 116 -/- p53, IMR 90 **(B)** MCF10A and 76N tert were cultured. Twenty-four hours later RNA was extracted and treated with DNase. Reverse transcription was carried out using the DNase treated RNA, followed by SYBR Green RT – PCR performed in triplicate to quantify the expression of both the isoforms (See Materials and Methods for details). Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to H1299, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

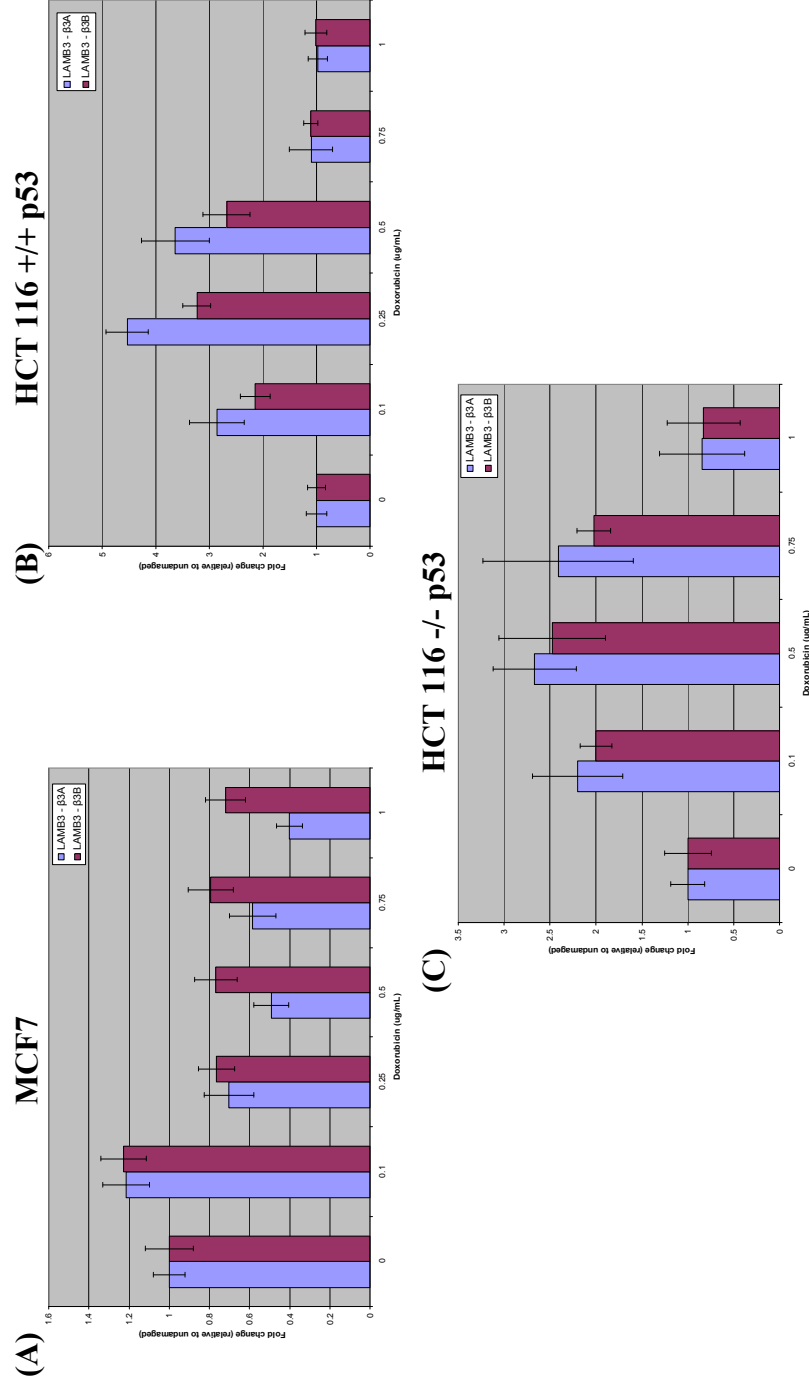


Figure 20: Levels of LAMB3 mRNA isoforms change upon Doxorubicin treatment. (A) MCF7, (B) HCT 116 +/+ p53, (C) HCT 116 -/- p53 cells were treated with increasing concentrations of the DNA damaging agent Doxorubicin. Twenty-four hours after treatment of doxorubicin, RNA was extracted and treated with DNase. Reverse transcription was carried out using the DNase treated RNA, followed by SYBR Green RT-PCR performed in triplicate to quantify the expression of the β3A and β3B isoform (See Materials and Methods for details). Both isoforms show a decrease in expression levels in MCF7 upon increase in doxorubicin doses, while they show a dose dependent pattern of expression in HCT 116 +/+ p53 and HCT 116 -/- p53 cells. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to undamaged, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

CELL LINE	SYBR Green PCR assay – DNA-damage by Doxorubicin (0 – 1 µg/mL)	
	LAMB3 isoforms	
	β3A	β3B
MCF7	Decrease	Decrease
HCT 116 +/+ p53	Increase	Increase
HCT 116 -/- p53	Increase	Increase

Table 7: - The two isoforms of LAMB3 show same response to Doxorubicin treatment. The table shows the response of the transcripts of two LAMB3 mRNA isoforms to Doxorubicin induced DNA-damage in MCF7, HCT 116 +/+ p53 and HCT 116 -/- p53 cell lines.

	MCF7			HCT 116 +/+ p53			HCT 116 -/- p53		
Doxorubicin (0.25 μg/mL)	RQ	RQ min	RQ max	RQ	RQ min	RQ max	RQ	RQ min	RQ max
0	1	0.91	1.09	1	0.86	1.16	1	0.82	1.23
0.1	1.30	1.12	1.51	2.49	2.17	2.85	2.03	1.81	2.28
0.25	0.70	0.64	0.77	3.96	3.61	4.34	N/A	N/A	N/A
0.5	0.59	0.46	0.75	2.74	2.35	3.20	2.69	1.62	4.48
0.75	0.78	0.66	0.94	1.56	1.35	1.81	2.23	1.53	3.26
1	0.60	0.53	0.67	1.00	0.81	1.25	0.78	0.45	1.35

Table 8: Combined levels of LAMB3 isoforms change upon Doxorubicin treatment. MCF7, HCT 116 +/+ p53 and HCT 116 -/- p53 were cultured. Twenty-four hours later RNA was extracted and treated with DNase. Reverse transcription was carried out using the DNase treated RNA, followed by SYBR Green RT–PCR performed in triplicate to quantify the combined expression of the isoforms (See Materials and Methods for details) using GAPDH as the normalizer. The combined transcript levels remained unchanged in MCF7 cells and increased in the other two cell lines. The Statistical significance was determined by calculating 95% confidence interval for the RQ value.

details) and LAMB3 transcript levels were evaluated upon the onset of oncogenic ras induced senescence. RNA extraction was done at the same time point when senescence was measured in cells by assessing the beta-galactosidase activity. RT-qPCR was employed to quantify LAMB3, p21 and p16INK4a (positive control for senescence) transcript levels. It was observed that LAMB3 and p21 transcript levels were up-regulated upon the onset of ras induced senescence (Figure 23A and 23B respectively). p16INK4a transcripts showed an increase in expression levels pointing to the fact that senescence was induced in the IMR90 cells (Figure 23C). Thus, I observed that an increase in LAMB3 transcript levels correlated with the accumulation of p53 which was seen by Serrano et. al. (Serrano et al., 1997) when oncogenic ras provoked premature cell senescence.

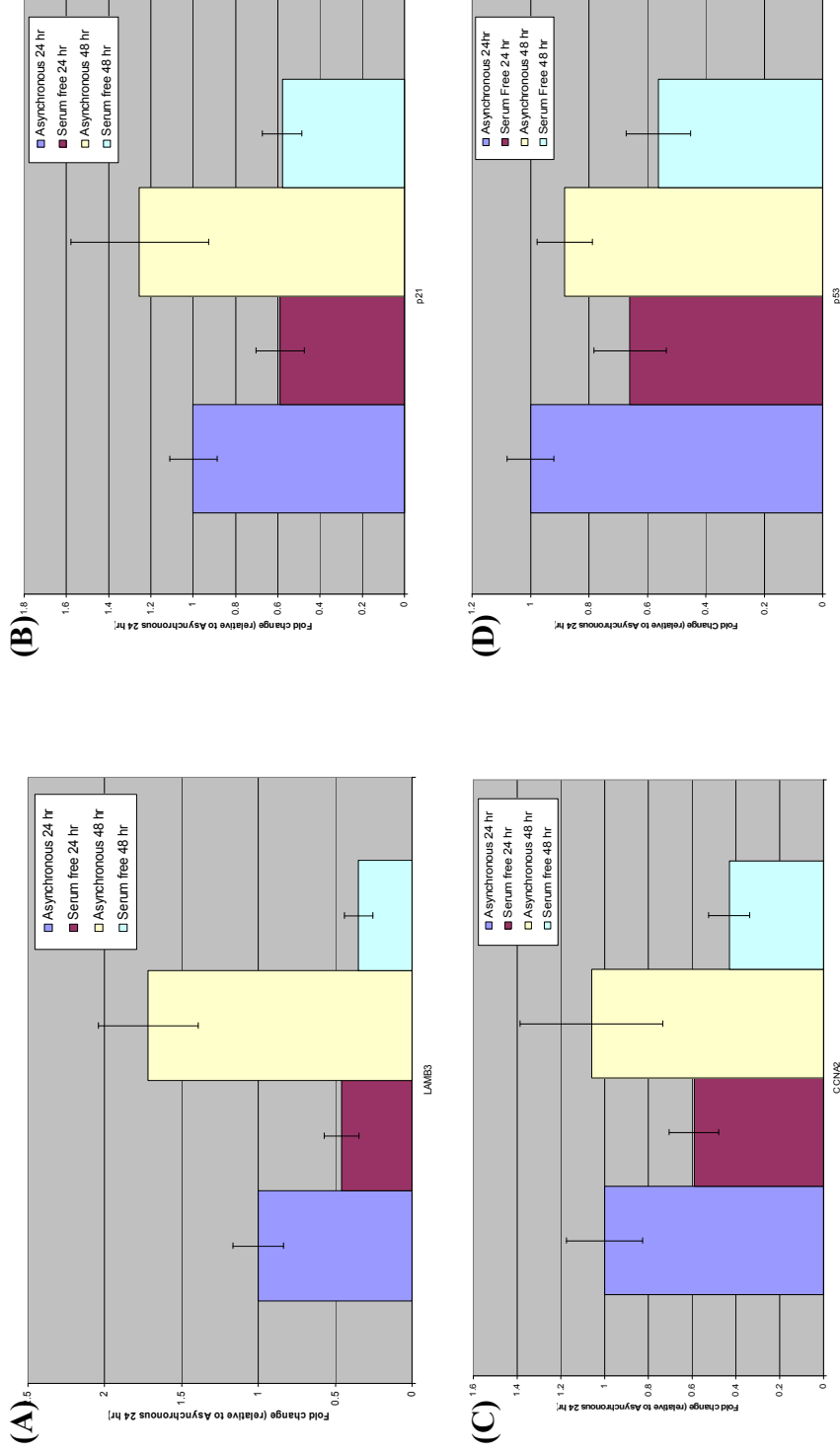


Figure 21: Serum deprivation in MCF7 cells. MCF7 cells were cultured and then serum deprived for 24 and 48 hrs (See Materials and Methods). RNA was extracted 24 and 48 hrs later in addition to 24 and 48 hrs after serum deprivation and Reverse transcription was performed. Following this, Real-time PCR was carried out in triplicate to quantify the expression of (A) LAMB3, (B) p21 (C) CCNA2 and (D) p53. Transcripts levels of each decreased upon serum deprivation. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to Asynchronous 24 hr, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars.

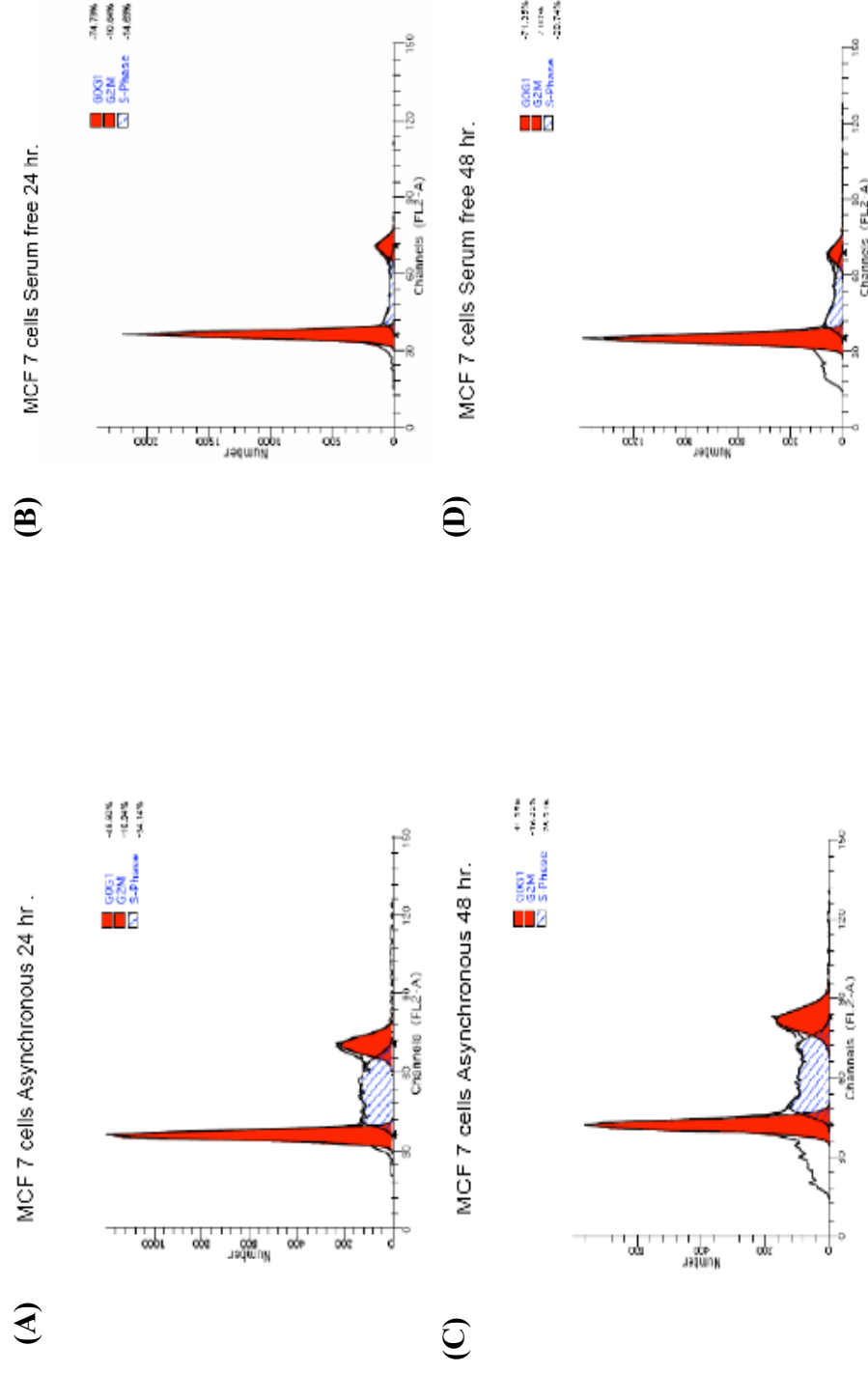


Figure 22: Serum deprivation in MCF7 cells for 24 and 48 hrs causes a G1 cell cycle arrest and apoptosis. MCF7 cells were cultured; serum deprived for 24 and 48 hrs, stained with propidium iodide and cell cycle distribution was determined by flow cytometry. Histogram indicates cell cycle progression for Asynchronous - (A) 24 hr population (C) 48 hr population and Serum Free - (B) 24 hr population and (D) 48 hr population. An increase in G1 and a decrease in S phase cells indicates a G1 cell cycle arrest while an increase in sub-G1 phase indicates apoptosis.

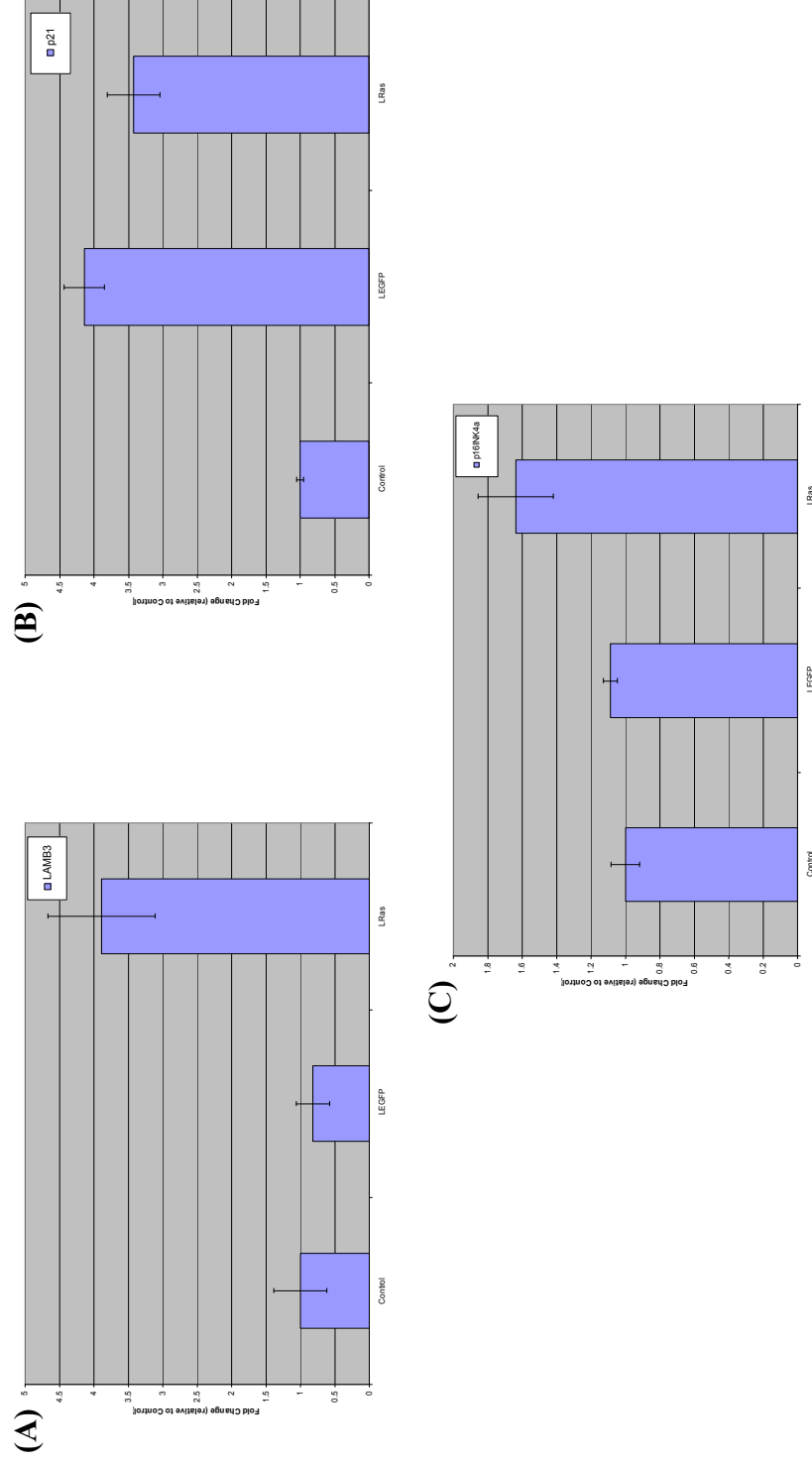


Figure 23: Ras induced senescence in IMR90 cells. IMR90 cells were transduced with the specific lentiviral vector. Senescence was assayed by measuring the β -Galactosidase activity (See Materials and Methods). RNA was extracted upon completion of 7 day treatment with the selection agent blasticidin followed by reverse transcription. Real-time PCR was then performed in triplicate to quantify the expression of (A) LAMB3, (B) p21 and (C) p16INK4a. Upon induction of senescence by Ras, increase in transcript levels for each was seen. Y axis is Relative Quantification (RQ) values, reported as the Fold change relative to Control, using GAPDH as the normalizer. The Statistical significance was determined by calculating 95% confidence interval for the RQ value, represented by the error bars. This experiment was performed by Kelly Miller and Meghna Jani.

IV. DISCUSSION

p53 is called as the “Guardian of the genome” because it functions by tightly controlling cell proliferation or inducing apoptosis. The ability of p53 to regulate a number of genes transcriptionally in response to stressors lies at the center of its function as a major tumor suppressor. The versatile and complicated mechanism by which p53 exerts its functions is slowly getting resolved as identification of many p53-regulated genes is taking place. In a broad spectrum of human cancers, inactivation of p53 is a crucial aspect of tumor formation (Vogelstein, Lane, & Levine, 2000). Considering its importance as a tumor suppressor, human tumors utilize various genetic mechanisms for inactivating the function of p53. Thus, it is important to study tumors wherein p53 is wild-type however one or both of its negative regulators i.e. Hdm2 and HdmX are overexpressed, leading to a non-functional p53. Taking this observation into account, our lab employed an RNAi approach targeting *hdm2* and *hdmX*. DNA microarrays were employed to examine how p53 is activated through loss of Hdm2 or HdmX altered gene expression. Data from this experiment not only revealed the effect of Hdm2 and HdmX knockdown on previously identified p53 target genes but also identified several potentially novel p53 regulated genes.

From the microarray experimental data it appeared that LAMB3 represented one such novel p53 regulated gene as LAMB3 mRNA levels increased following knockdown of Hdm2 and HdmX (Table 3). This information led to the formation of the hypothesis that LAMB3 is a novel and classical p53 target gene. Validation experiments were conducted to confirm the microarray analysis data, using RNAi against Hdm2 and HdmX in MCF7, MCF10A and 76N tert cells which led to an increase in LAMB3 transcript levels only for the Hdm2 + HdmX knockdown (Figure 3A, 4A and 5A). To further validate the possibility of LAMB3 being a p53 target gene, DNA damage experiments were performed on tumor and non-tumor cell lines wherein p53 was activated upon the induction of cellular stress. LAMB3 gene expression was unaffected in the two breast epithelial cell lines - MCF7 (breast cancer) and MCF10A (immortalized but non-transformed) upon DNA damage (Figure 7A and 8A respectively) but the expression of p21 (known p53 target and positive control) mRNA increased for each of the cell line (Figure 7B and 8B respectively). In contrast, HCT 116 +/+ p53 and HCT 116 -/- p53 – two colon cancer cell lines, showed an increased expression of LAMB3 transcripts (Figure 10A and 11A respectively) along with an increase in the p21 transcript levels (Figure 10B and 11B respectively) by DNA damage induced genotoxic stress. The increase in LAMB3 levels in a p53 null cell line upon DNA damage indicated that LAMB3 can be transactivated in a p53 independent manner upon cellular stress, which negates the possibility of it being a classical p53 target gene.

Interestingly, during the course of the DNA-damaging studies it was observed that not only LAMB3 transcript levels but also p21 transcript levels increase in the HCT 116 -

/- p53 cell line. Thus, it seems that other transcription factors can also regulate both these genes. This concept is supported by the recent observation whereby p21 expression is up-regulated upon DNA-damage in a cell line independent of p53. It was Ma et al., who reported that NFκB/p65 induces p21 in HCT 116 -/- p53 cells upon DNA-damage by doxorubicin (Ma et al., 2008). Hence, it will be exciting to analyze whether NFκB/p65 plays a role as a transcriptional regulator of LAMB3.

In an attempt to address other possible transcriptional regulators of LAMB3, I was also curious to look whether the p53 family members – p63 and p73 play a role in LAMB3 regulation. In a study by Vayssade et al., p73 was shown to activate p53 regulated genes following doxorubicin treatment of a p53-deficient breast cancer cell line (Vayssade et al., 2005). This observation supported the theory that there is a possibility of LAMB3 regulation by p63 and/or p73 in some manner. Hence, in-depth examination of the effect of both – p63 and p73 on LAMB3 expression will be fruitful.

In view of the above mentioned notion that LAMB3 expression might be regulated by p63 and p73, I performed overexpression studies whereby not only p63 and p73 but also p53 was overexpressed in four cell lines. From the results it was revealed that LAMB3 gene expression stands unchanged when p53, p63 or p73 were overexpressed, but, p21 transcript levels up-regulated for p53 and its family members (Figure 13 and 14). Thus, the results were not consistent with the assumption and led to the conclusion that LAMB3 expression is not transcriptionally regulated by

overexpression of p63 or p73. Since the overexpression of p53 also did not lead to any changes in LAMB3 transcript levels, hence LAMB3 does not fit the classical model to be considered as a p53 transcriptional target gene.

Taking into account the results of DNA damage and overexpression studies, LAMB3 does not portray the characteristics of a direct transcriptional target of p53, but there is a possibility of it being regulated by p53 through an indirect mechanism. Studies conducted in our lab had resulted in a cell cycle arrest upon loss of Hdm2 and HdmX in MCF7 cells (Heminger et al., manuscript submitted). Hence it could be possible that LAMB3 gene expression is regulated by cell cycle which in turn is influenced by p53 activity. Thus, to test this hypothesis, MCF7 cells were serum deprived, bringing about cell cycle arrest. It was observed that LAMB3 transcript levels were down-regulated upon induction of cell cycle arrest (Figure 21). p53 has been shown to play a role in cell cycle arrest and its protein levels were observed to be very low as cells underwent G1 phase arrest (Mosner et al., 1995; Reisman & Loging, 1998). Thus, the results of serum deprivation agree with the model that p53 maybe regulating LAMB3 indirectly. So, other ways of induction of cell cycle arrest should be tried (eg: - aphidocolin treatment) to check the effect on LAMB3 gene expression. Shen et al., saw a p53-independent cell cycle arrest in a human colon carcinoma cell line using the compound sulforaphane (Shen, Xu, Chen, Hebbar, & Kong, 2006). In view of this observation by Shen et al., examining the levels of LAMB3 after a p53-independent G1 cell cycle arrest will help in uncovering the p53 dependency in the regulation of LAMB3. Thus, it would be fruitful to

delve into understanding the factors involved in cell cycle arrest that would help illustrate the regulation of LAMB3.

Simultaneous to investigating the effect of cell-cycle arrest on LAMB3 gene expression it would be interesting to check whether the increased expression of LAMB3 in the validation experiments was p53 dependent. In this matter, both Hdm2 and HdmX have been found to possess p53 independent roles in tumor suppression (Ganguli & Wasyluk, 2003; Ma et al., 2008; Matijasevic, Steinman, Hoover, & Jones, 2008; Zhang et al., 2005). Hdm2 has also been shown to be involved in cell cycle arrest (Zhou, Frum, Deb, & Deb, 2005). Thus, it is possible that LAMB3 gene expression may be regulated by Hdm2 or HdmX in a p53-independent manner. To test this likelihood, RNAi targeting Hdm2 or HdmX in a p53 null cell line can be utilized as one of the ways to study the response of LAMB3 in the absence of p53. Additionally, I had observed high levels of LAMB3 protein in H1299 (p53 null) cells which have comparatively lower levels of Hdm2 and HdmX than MCF7 (wild-type p53 and no detectable LAMB3 protein) cells. Thus, if overexpressing Hdm2 or HdmX in a p53 null-cell line like H1299 (highly expressing LAMB3) causes a decrease in LAMB3 expression levels then it would allow us to pinpoint that Hdm2 or HdmX regulate LAMB3 in the absence of p53. These kinds of experiments will open a new path to identifying other possible regulators of LAMB3.

Along with looking at cell cycle as a mode of eliciting a non-genotoxic stress, I also examined LAMB3 response to oncogenic ras induced senescence, since senescence

has been demonstrated as a mode of tumor suppression (Sager, 1991). From the results it was seen that LAMB3 levels increased upon the onset of ras induced senescence along with the increase of p16INK4a transcript levels (Figure 23) which was used as a marker of senescence (Alcorta et al., 1996). Atadja et al. had reported increased activity of p53 in senescing fibroblasts (Atadja, Wong, Garkavtsev, Veillette, & Riabowol, 1995; Serrano, Lin, McCurrach, Beach, & Lowe, 1997) and hence, the increase in LAMB3 mRNA levels upon the onset of ras induced senescence could be a result of increased p53 activity. To prove that the increase in LAMB3 was dependent on p53 activation in this ras mediated senescence, additional experiments would need to be performed where either ras mediated senescence is induced in a p53 null cell line or RNAi is used to target p53 in cells where ras triggers senescence. In these experiments if there is no up-regulation of LAMB3 transcripts, it would imply the p53 dependency of LAMB3 expression. Also, studies by Lowe et al. indicate that oncogenic ras signals p53 leading to the induction of cellular senescence (Ferbeyre et al., 2002). To verify whether this condition is necessary to observe an increase in LAMB3 expression, mutant p53 should be introduced into cells, thereby blocking the p53 pathway. Separation of ras and p53 pathway in this manner will allow to pinpoint the requirement of not only p53 but also that of ras and senescence for up-regulating LAMB3 expression levels.

Although DNA-damage and overexpression experiments led to the conclusion that LAMB3 is not a classical p53 target, the knockdown, cell-cycle arrest and senescence experiments pointed out the possibility of LAMB3 being regulated in a non-genotoxic manner by p53. Thus, I wanted to check whether there exist any potential p53

response elements in or near the LAMB3 promoter. While searching for p53 response elements, four p53 half binding sites were seen in the first intron of LAMB3 (Figure 2B). In future, ChIP experiments could be performed to pinpoint whether p53 binds to LAMB3. Similarly, ChIP experiments following induction of genotoxic and non-genotoxic stresses in cells possessing wt-p53 and p53 null cells can further clarify the dependency of LAMB3 gene expression on p53.

Hao et al., (Hao, McDaniel, Weyer, Barrera, & Nagle, 2002) stated that both MCF7 and LnCap cell lines did not possess the protein producing mRNA isoform β 3A of LAMB3, thus questioning the conclusion derived from the LAMB3 response in several of the experiments performed. The SYBR Green PCR assay performed using primers specific for both isoforms of LAMB3 showed the presence of β 3A isoform in MCF7 but not in LnCap cells (data not shown). Thus, it is necessary to further evaluate absence of LAMB3 protein in MCF7 cells when the β 3A isoform of LAMB3 mRNA is present. Also, in-depth research on the reason behind absence of LAMB3 protein expression in some cell lines will help in better understanding the complicated LAMB3 response.

Taking all the findings into consideration, LAMB3 does not appear to be a classical target of p53 but maybe regulated by p53. Also, the validation experiments in the earlier course of studies opened up a new path to find novel p53 regulated genes. Elucidating possible novel p53 target genes will aid to improve diagnosis and treatment of cancer patients thus taking us a step further towards the 'Holy Grail' of p53 research.

V. APPENDIX

Appendix I

Isolation of Total RNA from Animal Cells (RNeasy[®] mini kit; Qiagen-Spin protocol)

1. Harvest cells (cells not more than 1×10^7 used) by aspirating the cell-culture medium and continue immediately with step 2.
2. Disrupt cells by addition of appropriate volume of Buffer RLT (see table below) to the cell-culture dish. Collect cell lysate with a rubber policeman. Pipet lysate into a micro centrifuge tube. Vortex or pipet to mix to ensure no cell clumps.

Dish diameter (cm)	Buffer RLT (μ L)
<6	350
6-10	600

3. Homogenize the sample by passing the lysate at least 5 times through a 20-gauge needle (0.9 mm diameter) fitted to an RNase-free syringe.
4. Add 1 volume (usually 350 μ L or 600 μ L) of 70% ethanol to the homogenized lysate, and mix well by pipetting. Do not centrifuge.

5. Apply up to 700 μL of the sample, including any precipitate that may have formed to an RNeasy[®] mini column placed in a 2 mL collection tube. Close the tube gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm). Discard the flow-through. If the volume exceeds 700 μL , load aliquots successively onto the RNeasy[®] column, and centrifuge as above. Discard flow-through after each centrifugation.
6. Add 700 μL Buffer RW1 to the RNeasy[®] column. Close the tube gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column. Discard the flow-through and collection tube.
7. Transfer the RNeasy[®] column into a new 2 mL collection tube. Pipet 500 μL Buffer RPE onto the RNeasy[®] column. Close the tube gently, and centrifuge for 15 s at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to wash the column. Discard the flow-through.
8. Add another 500 μL Buffer RPE to the RNeasy[®] column. Close the tube gently, and centrifuge for 2 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to dry the RNeasy[®] silica-gel membrane.
9. Place the RNeasy[®] column in a new 2 mL collection tube, and discard the old collection tube with the flow-through. Centrifuge in a microcentrifuge at full speed for 1 min.
10. To elute, transfer the RNeasy[®] column to a new 1.5 mL collection tube. Pipet 30-50 μL RNase free water directly onto the RNeasy[®] silica-gel membrane. Close the tube gently, and centrifuge for 1 min at $\geq 8000 \times g$ ($\geq 10,000$ rpm) to elute.

Appendix II

Synthesis of cDNA from total RNA by Reverse Transcription (Applied Biosystems-Protocol)

25 μ L reaction converts a maximum of 0.5 μ g of total RNA to cDNA.

RNA + water should be 9.62 μ L total and then add 15.38 μ L of the master-mix as shown below:

Component	Volume/tube (μ L) for one reaction	N + 1 reactions = Final concentration
RNA	As required	
RNase free water	9.62 – (RNA sample volume)	
10X Taqman RT buffer	2.5	1X
25 mM MgCl ₂	5.5	5.5 mM
DeoxyNTPs mixture	5	500 μ M per dNTP
Random hexamers	1.25	2.5 μ M
RNase Inhibitor	0.5	0.4 U/ μ L
Multiscribe reverse transcriptase (50 U/ μ L)	0.625	1.25 U/ μ L
Total	15.38	

Thermal cycling conditions are as follows:

Step	Incubation	Reverse Transcription	Reverse Transcriptase	
	HOLD	HOLD	HOLD	HOLD
Temperature	25°C	48°C	95°C	4°C
Time	10 min	30 min	5 min	Infinite
Volume	25 µL			

Store the cDNA sample at -15 °C to -25 °C

Appendix III

Senescence β -Galactosidase Staining kit (Cell Signaling technology™-protocol)

1. Remove the growth medium from the cells and wash the plate once with 2 mL 1X PBS.
2. Fix the cells with 1 mL 1X Fixative solution for 10-15 minutes at room temperature.
3. While the plate is in the Fixative solution, prepare the Staining solution. Use polypropylene plastic only.
 - a. 930 μ L Staining solution
 - b. 10 μ L Staining Supplement A
 - c. 10 μ L Staining Supplement B
 - d. 50 μ L 20 mg/mL X-gal in DMF
4. Wash the well twice with 2 mL 1X PBS.
5. Add 1 mL Staining solution mix to the plate. Incubate overnight at 37°C.
6. Check the cells under a microscope (200 x total magnification) for development of blue color.
7. For long-term storage of stained wells/plates, remove the Staining solution and overlay the cells with 70% glycerol. Store at 4°C

Appendix IV

DNase treatment of RNA (DNA-free kit; Ambion Inc. – protocol)

1. Add 0.1 volume 10X Dnase I Buffer and 1 μ L rDNase I to the RNA, and mix gently.
2. Incubate at 37°C for 20-30 min.
3. Add re-suspended DNase Inactivation Reagent (typically 0.1 volume) and mix well.
4. Incubate 2 min at room temperature, mixing occasionally.
5. Centrifuge at 10,000 x g for 1.5 min and transfer the RNA to a fresh tube.

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